PLP-dependent [alpha]-oxoamine synthases: Phylogenetic analysis, structural plasticity, and structure-function studies on 5-aminolevulinate synthase

Tracy D. Turbeville
University of South Florida

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PLP-Dependent α-Oxoamine Synthases: Phylogenetic Analysis, Structural Plasticity, and Structure-Function Studies on 5-Aminolevulinate Synthase

by

Tracy D. Turbeville

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Dedication

... to God who gave me the strength and perseverance to continue when I wanted to give up and who has filled my life with the love and support of many wonderful people.

...to my grandma, Anna Turbeville, who taught me the value of love and compassion. Her memory continues to be a source of inspiration and strength.

... and to my fiancée, Steve Colafrancesco, for his love, care, encouragement and support without which I would not have made it across the finish line.
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<td>AAT</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>Circular Dichroism</td>
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<td>2-Amino-3-Ketobutyrate Ligase</td>
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<td>MCD</td>
<td>Multiple Carboxylase Deficiency</td>
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<td>MP</td>
<td>Maximum Parsimony</td>
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<td>NAD$^+$</td>
<td>β-Nicotinamide Adenine Dinucleotide</td>
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<td>Porphobilinogen</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PLP</td>
<td>Pyridoxal 5’-Phosphate</td>
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<td>PMSF</td>
<td>Phenylmethylsulphonyl Fluoride</td>
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<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<td>SPT</td>
<td>Serine Palmitoyltransferase</td>
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PLP-Dependent $\alpha$-Oxoamine Synthases: Phylogenetic Analysis, Structural Plasticity, and Structure-Function Studies on 5-Aminolevulinate Synthase

Tracy Dawn Turbeville

Abstract

5-Aminolevulinate synthase (ALAS) and 8-amino-7-oxononanoate synthase (AONS) are two of four homodimeric members of the $\alpha$-oxoamine synthase family of pyridoxal 5'-phosphate (PLP)-dependent enzymes. The evolutionary relationships among $\alpha$-oxoamine synthases representing a broad taxonomic and phylogenetic spectrum have been examined to help identify residues that may regulate substrate specificity.

The structural plasticity of ALAS has been documented in studies of functional circularly permuted ALAS variants and the single polypeptide chain ALAS dimer (ALAS/ALAS) exhibiting a greater turnover number than wild-type ALAS. An examination of the contribution of each ALAS/ALAS active site to the enzymatic activity shows that each active site makes distinct contributions to the steady-state activity of the enzyme. Chimeric ALAS/AONS proteins exhibited an oligomeric structure with two sites having ALAS activity and two sites having AONS activity. Remarkably, the steady-state rates for both the ALAS and AONS activities were lower than that observed in the parent enzymes, while the reactivity of the ALAS sites in ALAS/AONS was similar to that of wild-type ALAS. We propose that the different contribution of each active site to the steady-state activity of ALAS/ALAS and the reduced steady-state
activities of the ALAS/AONS chimera, compared to the parent enzymes, relate to different extents of conformational changes associated with product release due to the strain caused with the linking the two ALAS (or ALAS and AONS) subunits. Thus, the extensive plasticity seen in ALAS extends to another member of the α-oxoamine family, AONS.

In the α-oxoamine synthase family a conserved histidine hydrogen bonds with the phenolic oxygen of PLP and may be significant for substrate-binding, PLP-positioning, and maintaining the pKₐ of the imine nitrogen. The replacement of this conserved histidine, H282, with alanine in murine erythroid ALAS has multiple effects on the spectral, binding, and kinetic properties of the enzyme and supports the conclusion that H282 plays multiple roles in the enzymology of ALAS. Altogether, these results imply that amino acid H282 coordinates the movement of the pyridine ring with the reorganization of the active-site hydrogen bond network and acts as a hydrogen bond donor to the phenolic oxygen to maintain the protonated Schiff base and enhance the electron sink function of the PLP cofactor.
Chapter One

Introduction

Historical Perspectives on Vitamin B₆

Cellular metabolism involves a series of multi-step reaction pathways which are necessary for the maintenance of life. Catabolic pathways break down substances to yield energy, whereas anabolic pathways assemble complex products. The diverse reaction pathways are intertwined with each pathway requiring a unique set of enzymes. Enzymes allow reactions to proceed rapidly and are essential to push thermodynamically unfavorable reactions by coupling them to favorable ones. While many of the enzymes involved in carrying out this vast array of chemical reactions do not need any additional components, some require a non-protein constituent or cofactor for activity. Cofactors can be inorganic or organic and many are essential for optimal health and disease prevention. Most vitamins are precursors for metabolically essential cofactors with diverse biochemical functions.

The wide availability of vitamins in the form of inexpensive pills has contributed to a modern day fascination with vitamin supplementation by many, making it hard to imagine that a century ago the existence of such “accessory food factors” was heavily debated. The first vitamin was isolated in 1913; by the mid 1930’s the first multivitamin products were available in pharmacies and grocery stores and milk was fortified with vitamin D. Today an average of 35% of Americans report the recent use of multivitamins
(Panel, 2006). Many diseases associated with vitamin deficiencies that were commonplace a century ago are virtually unknown to today’s generation.

Vitamin B₆ is among the many vitamins in which the nutritional importance has been recognized and the metabolic and biochemical significance has been well characterized. Vitamin B₆ is a collective term for a group of pyridine derivatives that serve as precursors for the coenzyme pyridoxal phosphate, which has been recognized as the cofactor for over 100 enzyme catalyzed reactions (Percudani and Peracchi, 2003). The scientific discoveries and developments leading to the isolation of vitamin B₆ are common to all vitamins.

**Discovery of vitamins**

“Hard work on interesting problems is enjoyable and preferable to aimless wasting of leisure time. It may also lead to unexpected findings that give insights into important related problems. Such unexpected findings—sometimes called ‘luck’—frequently happen to the active researcher, but only rarely to those who prefer talk to study and work. So one should study and work hard, on interesting problems of any nature, with the purpose of explaining nature and helping others (Miles and Metzler, 2004).”

*Esmond Snell*

In the last days of the 19th century, when Christian Eijkman was sent to the Dutch colony in Java to identify the microbe responsible for beriberi, a nervous system ailment that would eventually be linked to a deficiency of thiamine (vitamin B₁) in the diet, the link between diet and disease was almost unimaginable (Carpenter, 2003). During this period, the recent successes of Robert Koch in identifying microorganisms responsible for
several diseases had scientist around the globe searching for other links between disease and microorganisms (Carpenter, 2003). Eijkman attempted to replicate the disease and isolate the microbe by injecting blood from sick soldiers into animals, yet he was unable to replicate the disease or identify any associated microorganism. While Eijkman’s work did not find the source of beriberi, his rather serendipitous observation in the 1890’s would eventually link beriberi to dietary insufficiencies. He observed that “uninfected” chickens began exhibiting leg weakness (polyneuritis), a characteristic observed in beriberi patients. He carefully characterized the chickens’ illness, including their unexpected spontaneous recovery (Carpenter, 2003).

Eijkman soon began an inquiry to understand the chickens’ extraordinary recovery. He would learn that, prior to the appearance of the polyneuritis symptoms, the chickens’ diet was changed from unpolished brown rice to polished white rice and the recovery of the chickens coincided with the addition of brown rice back into the diet. After a series of carefully designed experiments, replicating the symptoms and recovery of the chickens, he confirmed that the observed polyneuritis was directly attributable to diet (Carpenter, 2003). Although Eijkman mistakenly reasoned that the white rice contained a toxin and the brown rice the antidote or “the anti-beriberi factor”, his inquiry into his chance observation would begin the transformation of the nutritional paradigm of the period. Eijkman’s health would prevent him from continuing his research and force him to return to Holland; however, he had laid the groundwork that led his successor and colleague, Gerret Grijns, to theorize in 1901 that white rice "lacked a certain substance of importance in the metabolism of the central nervous system"(Rosenfeld, 1997; Carpenter, 2003).
Sir Frederick G. Hopkins had begun his career during the same period as Eijkman when the prevailing nutritional paradigm was that calories alone were sufficient for animals to survive. His 1901 observation that mice lacking the amino acid tryptophan died within a “fortnight” (two weeks) would be Hopkins first challenge to this theory (Hopkins, 1912). Through a classic series of experiments, Hopkins unveiled evidence that there was an unknown constituent found in normal diets that was not found in a diet of purified proteins, carbohydrates, fats, and salts, in 1906 and 1907. However, it would be five years before Hopkins published his unorthodox findings in the 1912 article, "Feeding Experiments Illustrating the Importance of Accessory Food Factors in Normal Dietaries (Hopkins, 1912).”

By the time Hopkins research was finally published, other scientists had begun working to find the elusive “anti-beriberi” factor contained in the rice polishings. Among them was London chemist Casimir Funk, who believed he had isolated a nitrogen-containing factor that cured beriberi and assumed it to be an amine. Thus, Funk “suggested the name vitamine for it as being one of those nitrogenous substances, minute quantities of which are essential in the diet of birds, man and some other animals (Funk, 1912).” Although the anti-beriberi factor was only a contaminant and was eventually demonstrated to not be an amine, the name stuck (Carpenter, 2003).

As long as the nature of Hopkins and Funk dietary components remained elusive, the existence of “vitamines” would remain in doubt. Fortunately, a great deal of attention was focused on the “vitamine” question leading to the two groups simultaneously reporting the discovery of the first accessory food substance in the Journal of Biochemistry in 1913 (McCollum, 1913; Osborne, 1913). Both groups identified a fat-
soluble organic substance that was an essential nutritional component for rats. It was termed fat-soluble A and was the first dietary constituent to be recognized as a “vitamine.” (Rosenfeld, 1997)

As scientist began to distill the various fat soluble and water-soluble requirements, the number of essential nutritional components isolated and characterized grew. It soon became clear that these dietary factors were not in fact amines and subsequently the “e” was dropped. Thus the early nomenclature, fat soluble A and water soluble B and C, would eventually be shortened and referred to as vitamins A, B and C, respectively (Drummond, 1920).

While the nutritional significance of vitamins is widely understood today, in the early 20th century the mere suggestion that accessory dietary factors were important was revolutionary and controversial. Though neither Eijkman or Hopkins actually isolated the “anti-beriberi factor” or any “accessory food factors,” it was their observations and research that would ultimately led to the discovery of vitamins, thereby transforming nutritional research. The characterization of these trace nutrients not only revolutionized nutritional science, but contributed greatly to other scientific disciplines. In 1929, Eijkman and Hopkins would share the Noble prize in “Physiology or Medicine” for their work leading to the “discovery of growth-stimulating vitamins.” By 1965, nine other scientists would be awarded the Nobel Prize for their work with vitamins and another seven Nobel Laureates would make significant contributions to vitamin research.
Unraveling the Vitamin B complex

“[T]he history of B6 is a further proof that success usually is preceded by trials, tribulations, and recurrent disappointment. The most helpful factor, apart from perseverance and timeliness of the line of research, is the deliberate recognition of a principle that is paramount in scientific research; it is often almost beyond our control and touches closely on intuition. It is Walter B. Cannons’s ‘serendipity’ (Gyorgy, 1971).”

Paul Gyorgy, M.D.

Water-soluble vitamin B, the “anti-beriberi” factor, was initially believed to be a single compound. The 1919 JBC review by Mitchell was the earliest suggestion that vitamin B likely contained multiple compounds of nutritional significance (Mitchell, 1919; Carpenter, 2003). It was not until 1926 that Dutch scientists in Java isolated the first vitamin B component, thiamine, Grijns’ “anti-beriberi” factor (Jansen, 1926; Carpenter, 2003). Thiamine, also called Vitamin B1, would be the first B vitamin identified and the first vitamin to be recognized as an enzyme cofactor.

Seven additional chemically distinct components of the vitamin B complex would eventually be isolated and identified (Table 1.1). Ultimately, all eight would be identified either as an enzyme cofactor or an essential cofactor component. The discovery that vitamins functioned as enzyme cofactors demonstrated that vitamins play essential roles in various aspects of metabolism and began to bridge the gap between nutritional science and the then seemingly remote fields of cellular metabolism and enzymology.

Among the vitamin B components that would eventually illicit the attention of nutritionists, biochemists and enzymologists, was pyridoxine. First identified as a
substance present in a crude supplement that cured or prevented florid dermatitis in rats, pyridoxine was initially given the name vitamin B₆ by Paul Gyorgy (Gyorgy, 1934). This new vitamin was eventually isolated, synthesized, and ultimately given the name pyridoxine (Gyorgy, 1971). An amine and an aldehyde form of vitamin B₆, pyridoxamine and pyridoxal, respectively, was eventually identified by Esmond Snell, a biochemist studying microbial metabolism (Snell, 1942). Snell also identified the respective 5´-phosphate esters of pyridoxal, pyridoxal 5´-phosphate (PLP) as the major biological active form of the vitamin B₆ (Christen and Metzler, 1985).

**Characterization of Vitamin B₆ as a cofactor and coenzyme**

“Pyridoxal phosphate holds an exceptional place among the coenzymes with regard to both the unparalleled diversity of its catalytic function and to their paramount significance in biochemical transformations of amino acids and in integral nitrogen metabolism (Boyer, Lardy et al., 1960).”

*A. E. Braunstein*

Scientists studying vitamin B₆ began unraveling the significance of its role as a cofactor in cellular metabolism. Snell eventually demonstrated that heating pyridoxal with various amino acids would produce an oxo-acid and pyridoxamine in a reversible fashion, prompting the proposal that vitamin B₆ derivatives might be involved in enzymatic transaminations (Christen and Metzler, 1985). Almost simultaneously, tyrosine decarboxylase activity and enzymatic transamination between glutamate and oxaloacetate in *Streptococcus faecalis* cells were found to be dependent on the availability of pyridoxal by another group (Christen and Metzler, 1985).
However, it was the non-enzymatic catalytic activity of pyridoxal that would give the most insight into the magnitude of its role as a coenzyme. Pyridoxal demonstrated an ability to catalyze amino acid transaminations, racemization, and $\alpha, \beta$ eliminations (Metzler and Snell, 1952; Olivard, Metzler et al., 1952; Christen and Metzler, 1985; Miles and Metzler, 2004). The electron-withdrawing property of the heterocyclic nitrogen was ultimately recognized as a fundamental element to all pyridoxal-catalyzed reactions (Christen and Metzler, 1985). This led to a proposed mechanism based on the utilization of the electron withdrawing properties of the PLP-cofactor in the labilization of an $\alpha$-carbon bond of the amino acid substrate, which was eventually found to be common for both non-enzymatic and PLP-assisted enzymatic reactions (Christen and Metzler, 1985).

The catalytic diversity of PLP-dependent enzymes arises from modulation and enhancement of the intrinsic chemical properties of PLP by the active site environment. In PLP-dependent enzymes, the PLP forms an imine bond with the $\varepsilon$-amino group of an active site lysine; this Schiff base linkage is termed the “internal aldimine” and is outlined in Scheme 1.1. The reactions begin with a transaldimination in which the amino group of the amino acid substrate replaces the $\varepsilon$-amino of the lysine. This PLP-substrate complex is common to all reactions and is termed the “external aldimine”. Subsequently, the cleavage of an amino acid $\alpha$-carbon bond leads to a resonance stabilized quinonoid intermediate in which the extended pi-system of the coenzyme acts as an electron sink, storing electrons from the cleaved bond through the conjugated system of the Schiff base and pyridinium ring. Ultimately, the electrons are dispensed back to the $\alpha$-carbon
allowing the formation of new linkages (Christen and Metzler, 1985; Christen and Mehta, 2001).

The fact that this essential and ubiquitous coenzyme is associated with at least 145 different enzymatic activities attests to the versatility and demonstrates the biological importance of this cofactor (Percudani and Peracchi, 2003). PLP-dependent enzymes include amino acid racemases, transaminases, decarboxylases, synthases and aldolases. These enzymes play a key role in the synthesis, interconversion, and degradation of amino acids and are also involved in the synthesis of nucleic acids and protein cofactors such as NAD⁺, biotin and heme (Mehta and Christen, 2000).

**Medical significance of Vitamin B₆**

Vitamin B₆ would eventually be recognized by the medical field for being involved in significantly more bodily functions than any other nutrient, with over 100 essential biochemical reaction catalyzed by PLP-dependent enzymes in human metabolism (Tambasco-Studart, Titiz et al., 2005). A number of disorders have been associated with PLP-dependent enzymes, some of which are overviewed in table 1.2.

Because of the wide availability of vitamin B₆ in both plant- and animal-derived food, B₆ deficiencies are uncommon. However, several different mechanisms can lead to an increased requirement for vitamin B₆, including depletion by various drugs, renal dialysis, malabsorption, and hereditary errors in B₆ metabolism (Holman, 1995). With the wide range of reactions that are catalyzed by PLP-dependent enzymes, it is not surprising that vitamin B₆ deficiency has been associated with an array of symptoms including weakness, reduced resistance to infections, weight loss, depression, irritability,
sleeplessness, peripheral neuropathy, seborrhoea-like dermatosis regions, pellagra-like dermatitis, glossitis/stomatitis, however, weakness, sleeplessness, and depression are the most prominent effects (Holman, 1995).
<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Name</th>
<th>Deficiency effects</th>
<th>Cofactor</th>
<th>Function</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>Thiamin</td>
<td>Beri-beri</td>
<td>Thiamine Pyrophosphate</td>
<td>Activated aldehyde transfer</td>
<td><img src="image1" alt="Thiamin Structure" /></td>
</tr>
<tr>
<td>B2</td>
<td>Riboflavin</td>
<td>Ariboflavinosis</td>
<td>FMN and FAD</td>
<td>Redox reactions; Electron carrier</td>
<td><img src="image2" alt="Riboflavin Structure" /></td>
</tr>
<tr>
<td>B3</td>
<td>Niacin</td>
<td>Pellagra</td>
<td>NAD and NADP</td>
<td>Redox reactions; Electron Carrier</td>
<td><img src="image3" alt="Niacin Structure" /></td>
</tr>
<tr>
<td>B5</td>
<td>Pantothnic Acid</td>
<td>Paresthesis</td>
<td>Coenzyme A</td>
<td>Acyl group transfer</td>
<td><img src="image4" alt="Pantothnic Acid Structure" /></td>
</tr>
<tr>
<td>B6</td>
<td>Pyridoxine</td>
<td>Various symptoms</td>
<td>Pyridoxal Phosphate</td>
<td>Amino Acid Metabolism: Transaminations, Deaminations, Decarboxylations, Condensations</td>
<td><img src="image5" alt="Pyridoxine Structure" /></td>
</tr>
<tr>
<td>B7</td>
<td>Biotin</td>
<td>Infants- Impair growth; neurological disorders</td>
<td>Biotin</td>
<td>Carrier of activated CO₂</td>
<td><img src="image6" alt="Biotin Structure" /></td>
</tr>
<tr>
<td>Vitamin</td>
<td>Name</td>
<td>Deficiency effects</td>
<td>Cofactor</td>
<td>Function</td>
<td>Structure</td>
</tr>
<tr>
<td>--------</td>
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<td>-----------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>B9</td>
<td>Folic Acid</td>
<td>Macrocyclic anemia; Birth defects</td>
<td>Tetrahydrofolate</td>
<td>One carbon transfer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B12</td>
<td>Cobalamin</td>
<td>Pernicious anemia</td>
<td>Adenosylcobalamin;</td>
<td>Intramolecular rearrangements; Methylation;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methycobalamin</td>
<td>Ribonucleotide reduction</td>
<td></td>
</tr>
</tbody>
</table>
SCHEME 1.1 - Reactions of PLP-dependent enzymes
<table>
<thead>
<tr>
<th>PLP dependent enzymes</th>
<th>Function</th>
<th>Associated Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine aminotransferase</td>
<td>Interconversion of alanine and pyruvate</td>
<td>Hyperoxaluria type I</td>
</tr>
<tr>
<td>Aminolevulinate synthase</td>
<td>Heme biosynthesis</td>
<td>X-linked sideroblastic anaemia</td>
</tr>
<tr>
<td>Cystathionine b-Synthase</td>
<td>Clearance of intracellular homocysteine</td>
<td>Hereditary homocystinuria and increase risk for atherosclerotic, cardio-, cerebro- and peripheral vascular diseases, and deep vein thrombosis and thromboembolism</td>
</tr>
<tr>
<td>Cystathionine γ-lyase</td>
<td>Trans-Sulfuration L-cystathionine to L-cysteine, α-ketobutyrate and ammonia</td>
<td>Cystathioninuria, cystinosis and homocystinuria</td>
</tr>
<tr>
<td>DOPA decarboxylase</td>
<td>formation of dopamine decarboxylation of L-aromatic amino acids into corresponding amines</td>
<td>Parkinson's disease and hypertension</td>
</tr>
<tr>
<td>GABA transaminase</td>
<td>Breakdown of GABA, regeneration of glutamate</td>
<td>GABA inhibition/glutamate excitation</td>
</tr>
<tr>
<td>Glutamate decarboxylase</td>
<td>Conversion of glutamate to GABA</td>
<td>Low GABA levels implicated in the symptoms associated with epilepsy, Parkinson's disease, Huntington's chorea, Alzheimer's disease and tardive dyskinesia</td>
</tr>
<tr>
<td>Histidine decarboxylase</td>
<td>Synthesis of histamine</td>
<td>Inflammatory diseases, some neurological and neuroendocrine disorders, osteoporosis and several types of neoplasias</td>
</tr>
<tr>
<td>Ornithine d-aminotransferase</td>
<td>Initial and rate-limiting step in the biosynthesis of polyamines</td>
<td>Ornithine anemia with gyrate atrophy</td>
</tr>
<tr>
<td>Serine hydroxymentyl transferase</td>
<td>One-carbon metabolism (methionine and lipids biosynthesis)</td>
<td>Suspected increased risk of lung cancer</td>
</tr>
<tr>
<td>Serine palmitoyltransferase</td>
<td>Sphingomyalin synthesis</td>
<td>Hereditary sensory neuropathy type I</td>
</tr>
</tbody>
</table>
**Vitamin B₆-dependent enzymes**

Reactions catalyzed by PLP-dependent enzymes play key roles linking different metabolic pathways and are thought to have been essential in the last universal ancestor of contemporary cells, in which the major metabolic pathways were likely established (Ouzounis and Kyrpides, 1996; Mehta and Christen, 2000). Thus, it has been proposed that this class of enzymes were already present in a universal progenitor cell 1000 to 1500 millions of years ago (Mehta and Christen, 1998). The sequence similarities among this highly divergent class of enzymes have generally been too low to establish a common ancestry. However, after considerable progress was made in the three-dimensional structure determination of a representative number of PLP-dependent enzymes, Christen and Mehta were finally able to use the high structural conservation of PLP-dependent enzymes to verify an evolutionary relationship (Christen and Mehta, 2001).

Initially, PLP-dependent enzymes were classified according to reaction specificity relative to the Cα, but as the database of PLP-enzyme structures grew it became possible to classify according to fold-types derived from three-dimensional structures (Alexander, Sandmeier et al., 1994; Christen and Mehta, 2001; Eliot and Kirsch, 2004). There are 4 fold-types of PLP-dependent enzymes and each corresponds to an independent evolutionary lineage. The aspartate aminotransferase superfamily corresponds to the fold-type I, α–family is the largest and most diverse lineage of PLP-dependent enzymes, of which the members outnumber the combined total of the other families (Mehta and Christen, 1998; Mehta and Christen, 2000; Paiardini, Bossa et al., 2004).
The high structural conservation displayed by the \( \alpha \)-family, despite a low degree of sequence identity (1.1), is not unusual among proteins sharing a common ancestor (Paiardini, Bossa et al., 2004). The dimer is the minimal functional unit for this family and each monomer consists of three domains (1.2), a short N-terminal domain (~50 residues), a central catalytic core (~250 residues), and a C-terminal domain (~100 residues) (Alexeev, Alexeeva et al., 1998; Astner, Schulze et al., 2005; Yard, Carter et al., 2007). Each dimer contains two identical active sites which lie at the interface between the two subunits. Both active sites interact with the PLP cofactor through a Schiff base linkage with an active site lysine (Christen and Mehta, 2001). In all known structures of the \( \alpha \)-family, the pyridine ring of the PLP cofactor is nearly superimposable (Kack, Sandmark et al., 1999). The pyridoxal moiety interacts with the enzyme in a common motif, which also includes a salt-bridge between the pyridinium ring nitrogen and an aspartate, and a hydrogen bond with the phenolic oxygen which occurs through a variety of amino acids in addition to Schiff base linkage with an active site lysine (Kack, Sandmark et al., 1999). Interestingly, the active site lysine and aspartate are the only two perfectly conserved amino acids in the \( \alpha \)-family (Mehta and Christen, 1998).

The \( \alpha \)-oxoamine synthases are a small group of enzymes within the \( \alpha \)-family that catalyze reactions between amino acids and CoA thioesters. 5-Aminolevulinate synthase (ALAS) and 8-amino-7-oxononanoate synthase (AONS) belong to this \( \alpha \)-oxoamine synthase subfamily, along with serine palmitoyltransferase (SPT) and 2-amino-3-oxobutyrate CoA ligase (KBL) (Alexeev, Alexeeva et al., 1998; Schneider, Kack et al., 2000; Schmidt, Sivaraman et al., 2001; Astner, Schulze et al., 2005; Yard, Carter et al.,
2007). These four members of the \( \alpha \)-oxoamine family share about 12% identity, with the sequence identity around 30% between any pair (Alexeev, Alexeeva et al., 1998).

ALAS, AONS and SPT catalyze condensations between amino acids and acyl-CoA thioesters with the concomitant decarboxylation of the amino acid, while KBL only catalyzes the condensation between the acyl-CoA thioester and the amino acid (Schneider, Kack et al., 2000). ALAS and AONS function in the biosynthesis of prosthetic groups essential for proteins that play fundamental roles in many biochemical processes. ALAS catalyzes the first committed and rate limiting step of heme biosynthesis in non-plant eukaryotes and the alpha subdivision of purple bacteria (Jordan, 1991) and AONS catalyzes the first committed step in biotin biosynthesis (Alban, Job et al., 2000).
FIGURE 1.1
Ribbon diagrams of representative enzymes of Fold Types I - IV. Each structure depicts a homodimer with the individual monomers distinguished by color. A. Fold-type I (aspartate aminotransferase family), 2-amino-3-oxobutyrate CoA ligase, 1FC4, B. Fold-type II (tryptophan synthase family), tryptophan synthase, 1EX5, C. Fold-type III (alanine racemase family), Alanine racemase, 1FST, D. Fold-type IV (D-amino acid aminotransferase family), branch chain aminotransferase, 1KTA, (In A,B and E the PLP cofactor is shown in red, while in C the active site lysine is shown in red.) Images were generated with Deep View (Guex and Peitsch, 1997; Kaplan and Littlejohn, 2001).
FIGURE 1.2
PLP-dependent enzymes of the α-family are homodimers. The ALAS homodimer from *R. capsulatus* (PDB code: 2BWN) in ribbon representation with one subunit shown in yellow and the central catalytic core, N-terminal domain, and C-terminal domain of the second subunit rendered in dark, medium and light blue, respectively. Depicted in ball-and-stick are the PLP cofactor (*red*), the active site lysine (*green*) involved in the Schiff base linkage with PLP and the active site residue F342 (*purple*), which is contributed by the adjacent chain. Image was generated with Deep View (Guex and Peitsch, 1997; Kaplan and Littlejohn, 2001).
Biotin

Biotin Biosynthesis

Biotin (Vitamin H or B7) is a cofactor for a small number of carboxylases and decarboxylases and is essential for life in all organisms (Scheme 1.2) (Alban, Job et al., 2000). Biotin biosynthesis occurs in most bacteria and archea as well as some fungi and plants. Animals and most fungi are biotin auxotrophs (Alban, Job et al., 2000; Streit, W. R. and Entcheva, P., 2003). The first common precursor in biotin biosynthesis is pimeloyl-CoA (Alban, Job et al., 2000; Streit, W. R. and Entcheva, P., 2003). The precursors for pimeloyl-CoA are not uniform and are not known for all microbes that synthesize biotin. In plants and gram-positive bacteria, pimeloyl CoA is synthesized from pimelic acid by pimeloyl-CoA synthetase. The four steps that convert pimeloyl-CoA to biotin are conserved among all organisms known to produce biotin (Alban, Job et al., 2000; Streit, W. R. and Entcheva, P., 2003).

The first enzyme of the common pathway is AONS, which catalyzes the decarboxylative condensation of alanine and pimeloyl-CoA in the first committed step of biotin biosynthesis, Scheme 1.3 (Schneider, G. and Lindqvist, Y., 2001). The next step is the conversion of 8-amino-7-oxopelargonic acid to 7, 8-diaminopelargonic acid (DAPA) catalyzed by DAPA aminotransferase. Both AONS and DAPA aminotransferase belong
to the α-family of PLP-dependent enzymes. DAPA aminotransferase is the only known aminotransferase that uses s-adenosylmethionine (SAM) as the NH$_2$ donor (Kack, Sandmark et al., 1999; Schneider, G. and Lindqvist, Y., 2001). In the following reaction, which is catalyzed by dethiobiotin synthetase, an uriedo ring is formed via a carboxylation reaction and requires ATP, Mg$^{2+}$ and CO$_2$ (Schneider, G. and Lindqvist, Y., 2001). The final step of biotin biosynthesis involves the insertion of a sulfur atom between the non-reactive methyl and methylene carbon atoms adjacent to the ureido ring of dethiobiotin (Schneider, G. and Lindqvist, Y., 2001) and is catalyzed by biotin synthase which functions as a homodimer. Biotin synthase is a s-adenosylmethionine dependent-enzyme with one 2Fe-2S cluster per monomer when isolated anerobically (Schneider, G. and Lindqvist, Y., 2001; Lotierzo, Tse Sum Bui et al., 2005). There is disagreement surrounding the identity of the sulfur donor, while free cysteine has been the generally accepted donor, recent studies indicate that the Fe-S cluster may be the true sulfur source (Schneider, G. and Lindqvist, Y., 2001).

**Medical significance of biotin**

Biotin (Vitamin H or B$_7$), another component of the vitamin B complex isolated by the physician Paul Gyorgy, is a cofactor for a small number of carboxylases and decarboxylases (Alban, Job et al., 2000). Biotin-dependent carboxylases add carbon dioxide to substrates and require ATP hydrolysis. The general mechanism for biotin dependent carboxylases is shown in Figure 1.3. Biotin-dependent carboxylases play essential roles in the metabolism of cholesterol, amino acids, and leucine degradation (Pacheco-Alvarez, Solorzano-Vargas et al., 2002).
In humans, there are five biotin-dependent carboxylases: propionyl-CoA-, β-methylcrotonyl-CoA-, two forms of acetyl-CoA and pyruvate carboxylase (Pacheco-Alvarez, Solorzano-Vargas et al., 2002). The absolute requirement for biotin rests in the central role that these four enzymes play in cell metabolism, which is summarized in Figure 1.4. The two most notable examples of biotin dependent-enzymes are acetyl-CoA carboxylase and pyruvate carboxylase. Acetyl-CoA carboxylase catalyzes the production of malonyl-CoA from acetyl-CoA, the first and rate-limiting step in fatty acid synthesis (Pacheco-Alvarez, Solorzano-Vargas et al., 2002). Pyruvate carboxylase is an anaplerotic enzyme involved in the formation of oxaloacetate from pyruvate, and oxaloacetate is a key intermediate in both gluconeogenesis and the TCA cycle (Pacheco-Alvarez, Solorzano-Vargas et al., 2002).

Humans, like other animals, are unable to synthesize biotin and depend on diet to fulfill their need for this vitamin. The primary source of dietary biotin is protein-bound and biotin must be cleaved. Pancreatic biotinidase cleaves the protein-bound biotin from the ε-amino group of the lysine residue to which it is attached (Suzuki, Aoki et al., 1994). Higher organisms have evolved an efficient biotin cycle to survive with the low concentrations of biotin found in natural food sources (Pacheco-Alvarez, Solorzano-Vargas et al., 2002). During carboxylase turnover, biotin is cleaved by cytosolic plasma and biotinidase (Gravel and Narang, 2005). Once the biotin is inside the cell, holocarboxylase synthetase covalently attaches biotin to carboxylases (Gravel and Narang, 2005).

Both biotinidase and holocarboxylase synthase deficiencies result in Multiple Carboxylase Deficiency, MCD. The symptoms for MCD include; alopecia,
developmental delay, organic aciduria, seizures, skin rashes, mild hyperammonemia, and breathing problems. Because biotinidase deficient patients only lack the ability to generate free biotin, it is easily treated by biotin supplementation (5–20 mg per day) (Leon-Del-Rio and Gravel, 1994; Suzuki, Aoki et al., 1994). In holocarboxylase synthetase deficient patients, carboxylase biotinylation is compromised globally, resulting in lethal consequences if not diagnosed and treated rapidly. Since holocarboxylase synthase deficiency leading to MCD is due to mutations resulting in decreased biotin affinity, the addition of pharmacologic doses of biotin (10–100 mg per day) to the diet can reverse the symptoms if treated promptly (Pacheco-Alvarez, Solorzano-Vargas et al., 2002).
SCHEME 1.3  Biotin Biosynthesis
Biotin-dependent carboxylases. A similar catalytic mechanism is shared by biotin-dependent carboxylases. Most biotin-dependent enzymes have three functional domains, the biotin carboxylase (or decarboxylase) domain, the carboxyltransferase domain and the biotin carboxyl carrier domain. The reaction occurs in two steps in two separate subsites. The first step occurs at the first subsite on the carboxylase domain and involves the partial fixation of CO$_2$ to biotin. The carboxybiotin then swings to the second subsite on the carboxyltransferase domain where a carboxylated compound is formed with the carboxylases or the generation of free CO$_2$ for the decarboxylases (Jitrapakdee and Wallace, 2003).
FIGURE 1.4: Biotin-dependent carboxylases in metabolism
Tetrapyrroles are indispensable to various biological processes, including oxygen transport (heme), photosynthesis (chlorophyll), electron transport (cytochromes), methionine and methylmalonyl CoA synthesis (colabalamin) and nitrate reduction (siroheme) (Frankenberg, Moser et al., 2003). The universal precursor of heme (Scheme 1.4) and other tetrapyrroles is 5-aminolevulinic acid, ALA. There are two distinct pathways leading to ALA formation and both require PLP. The C5 pathway, found in plants, algae, and most bacteria, utilizes the 5C-skeleton of glutamate and involves the action of two enzymes, the NADPH-dependent glutamyl-tRNA reductase (GluTR) and the PLP-dependent glutamate-1-semialdehyde-2,1-aminomutase (GSAM) (Frankenberg, Moser et al., 2003). The Shemin, or C4 pathway, identified in non-plant eukaryotes and the α-subdivision of purple bacteria requires a single PLP-dependent enzyme, 5-
aminolevulinate synthase (ALAS) (Shemin, Russell et al., 1955). The Shemin pathway for heme biosynthesis begins with the condensation of succinyl-CoA and glycine to produce carbon dioxide, CoA and ALA (Shemin, Russell et al., 1955; Ryter and Tyrrell, 2000).

In humans and other mammals, heme biosynthesis has been well characterized and begins in the mitochondria with the biosynthesis of ALA (Thunell, S., 2000). The prime location of heme biosynthesis occurs in the bone marrow and liver, however, the mechanisms controlling synthesis differ. Because heme demands vary significantly according to the unique requirements of different tissues, it is not unexpected that there is not a ubiquitous regulatory pathway. While the major control of heme biosynthesis in all cells is the production of the initial precursor ALA by ALAS, regulation of ALAS varies in various cell types (Thunell, S., 2000).

Two cytosolic ALAS isoenzymes are encoded by separate genes; the general housekeeping form, ALAS-1, and the erythroid specific form ALAS-2 and each are regulated by different mechanisms. The housekeeping isoenzyme, ALAS-1, has a short half-life and can be rapidly turned over in response to the drain of the free cellular heme pool by present metabolic needs (Thunell, S., 2000). The erythroid isoenzyme, ALAS-2, regulation machinery is designed for uninterrupted production and induced only during the period of active hemoglobin synthesis. ALAS-2 is regulated transcriptionally and post-transcriptionally by erythropoietin action and the amount of free iron present (Thunell, S., 2000; Zoller, Decristoforo et al., 2002). The regulation of ALAS-2 by intracellular free iron involves a mRNA stem loop structure in the 5’ untranslated region which contains an iron responsive element (IRE). The binding of iron regulatory
proteins, IRP-1 and IRP-2, to the IRE inhibits ALAS-2 mRNA translation under iron-poor conditions. High intracellular iron availability induces the post-translational modification of IRP-1 and degradation of IRP-2 to permit ALAS-2 mRNA translation.

ALA then moves into the cytosol, where ALA dehydratase (or porphobilinogen synthase) catalyzes the condensation of two ALA molecules to form the pyrrole ring of porphobilinogen, Scheme 1.5 (Thunell, S., 2000). Four porphobilinogen molecules are polymerized by porphobilinogen deaminase to form the linear tetrapyrrole of hydroxymethylbilane, which is then cyclized to uroporphyrinogen III by uroporphyrinogen III synthase (Frankenberg, Moser et al., 2003). The decarboxylation of 4 acetate groups of uroporphyrinogen III by uroporphyrinogen III decarboxylase yields coproporphyrinogen III (Ferreira, 2004). Heme biosynthesis then moves back into the mitochondria where two propionate side chains are oxidized by coproporphyrinogen III oxidase to yield protoporphyrinogen IX (Ferreira, 2004). Protoporphrinogen IX oxidase then catalyzes a six electron oxidation completing the conjugated system of the tetrapyrrole microcycle yielding protoporphyrin IX (Ferreira, 2004). In the final step of heme biosynthesis, iron is inserted into protoporphyrin IX by ferrechelatase to produce heme (Ferreira, 2004).

While the biosynthesis of the different tetrapyrroles can require between 7 and 30 reactions, the three reactions following ALA biosynthesis leading to uroporphyrinogen III found in heme biosynthesis are common for all tetrapyrroles (Figure 1.5). The steps leading from uroporphyrinogen III to protoporphyrin IX are common to both heme and chlorophyll biosyntheses. In plants, the major site of tetrapyrrole biosynthesis is in the plastids, although there is some debate regarding biosynthesis of heme in the
mitochondria. Specifically, the co-localization of the three terminal enzymes of heme biosynthesis may occur in both the plastid and the mitochondria, however there is not consensus among researchers (Tanaka and Tanaka, 2007).
SCHEME 1.5 Heme Biosynthesis
FIGURE 1.5: Overview of tetrapyrroles biosynthesis
Medical significance of hemoproteins and heme biosynthesis

In humans, the role of heme as a prosthetic group for the erythrocyte oxygen transporter protein hemoglobin is, perhaps, the most commonly known role. In fact, 85% of the body’s heme is synthesized in the erythroblast. While hemoglobin is found exclusively in erythrocytes, other specialized globins also function as oxygen carriers in fetal erythrocytes, neurons, cardiac muscle, and skeletal muscle. Once molecular oxygen is captured by the heme prosthetic group in hemoglobin, it can be transported via the bloodstream to the other oxygen chaperones, such as myoglobin in muscle cells, where ultimately it can be used as an electron acceptor. Impaired globin synthesis is associated with thalassemia syndromes and sickle cell disease is caused by mutations in the β-globin gene (Cecil, Goldman et al., 2008).

In addition to the ability of heme to interact with oxygen, heme can readily convert between oxidation states, Fe$^{2+}$ and Fe$^{3+}$, allowing heme to function as an electron carrier that may alternatively be reduced or oxidized. This characteristic makes heme an essential cofactor for a number of enzymes that catalyze redox and single electron carrier reactions during cellular processes such as cellular respiration, steroid metabolism and oxidative metabolism of foreign compounds such as drugs. The role of hemoproteins in various biological processes is summarized and examples are given in Table 1.3. Heme can also function as a regulatory molecule in some transcription factors and proteins involved in various biochemical pathways by controlling the activity by binding “heme regulatory motifs” (Rodgers, 1999).

Cytochrome P450s (CYPs) are a large class of hemoproteins and at least 27 functioning human CYPs have been identified (Nelson, Zeldin et al., 2004). CYPs
utilize the redox and electron carrier potential of heme and are involved in a variety of reactions. CYPs play key roles in cholesterol homeostasis and in the metabolism of drugs and environmental chemicals (Agundez, 2004). CYP gene polymorphisms have been associated with cancer (Agundez, 2004) and altered regulation of CYPs occurs in diseases such as obesity, diabetes, and non-alcoholic steatohepatitis (Luoma, 2008).

The interruption of heme biosynthesis is also associated with a variety of diseases which include X-sideroblastic anemia and porphyrias. Sideroblastic anemias are a group of hematological disorders which is characterized by microcytic hypochromic anemia with ringed sideroblasts in bone marrow (Bottomley, May et al., 1995). X-linked sideroblastic anemia, an inherited form of sideroblastic anemia, is due to any number of mutations in the erythroid-specific ALAS2 gene located on the X-chromosome (May and Bishop, 1998). In addition to the symptoms associated with anemia, patients also exhibit iron overload resulting from increased intestinal absorption of dietary iron and result in the major complications of this disorder (Bottomley, 1982; Propper and Nathan, 1982). The increased iron delivery to the tissues ultimately leads iron overload and secondary hemochromatosis which can result in diabetes, liver and heart failure without treatment (Bottomley, 1991; May and Bishop, 1998).

Porphyrias are a group of diseases associated with the accumulation of porphyrin intermediates of heme biosynthesis. Porphyrias are classified as hepatic or erythropoietic and neurovisceral or photocutaneous, according to the site of expression and the associated symptoms, although some overlap, Table 1.4. A wide array of neurovisceral and/or photocutaneous symptoms is associated with porphyrias. In addition to porphyrin neurotoxicity, the neurovisceral symptoms are also thought to be associated with the
build up of ALA or PBG, which also have a role neurotoxicity (Ryter and Tyrrell, 2000; Dombeck, T. A. and Satonik, R. C., 2005). Furthermore, ALA may act as a GABA analog and interact with neural GABA receptors (Dombeck, T. A. and Satonik, R. C., 2005). Photocutaneous symptoms are caused by porphyrin activation with long wave UV light and the subsequent generation of oxygen radicals that damages the skin (Dombeck, T. A. and Satonik, R. C., 2005).

There are 8 types of porphyrias which are associated with the 8 enzymes involved in heme biosynthesis. The recently characterized X-linked-dominant protoporphyria results from gain-of-function mutations in the rate-limiting ALAS2 (Whatley, Ducamp et al., 2008). The increased ALA production is propagated down the pathway resulting in an overproduction of protoporphyrin, which ultimately results in a disparity between the protoporphyrin and heme production. This porphyria results in photosensitivity indistinguishable from erythropoietic protoporphyria which is associated with impaired ferrochelatase activity, with almost one-fifth of the cases exhibiting liver disease (Whatley, Ducamp et al., 2008).

ALA dehydratase porphyria (AD-P) is the rarest, with only four confirmed cases (Sassa and Kappas, 2000). As the name suggests, this porphyria is attributed to a deficiency in ALA dehydratase. AD-P is autosomal recessive and is classified as an acute hepatic porphyria (Sassa and Kappas, 2000). A major characteristic found in patients is a marked increase in the urinary excretion of ALA (Sassa and Kappas, 2000).

Acute intermittent porphyria (AIP) is an autosomal dominant hepatic porphyria that is the most significant with respect to its incidence and clinical severity (Sassa and Kappas, 2000; Thunell, S., 2000). AIP results from a deficiency in porphobilinogen
deaminase in which the porphobilinogen deaminase activity is reduced by approximately 50% (Sassa and Kappas, 2000). The majority of those with AIP are clinically latent and only around one-half will experience an attack during their life span. The acute attacks occur in about 1-2 individuals per 100,000 in the US and in certain populations, such as Lapland, Sweeden, the rate may be as high as 1/1000 (Dombeck, T. A. and Satonik, R. C., 2005). Acute attacks may be life threatening and are often precipitated by various factors such as drugs, hormones, starvation, and infection (Dombeck, T. A. and Satonik, R. C., 2005). These factors induce heme synthesis by either depleting heme stores or via more direct mechanisms of ALAS induction. This results in an increase in porphyrin intermediates in the heme pathway. Patients exhibit a variety of visceral and peripheral symptoms from the involvement of the autonomic and central nervous system during an attack (Thunell, S., 2000; Dombeck, T. A. and Satonik, R. C., 2005). Extreme abdominal pain, tachycardia, nausea and various other neuropsychiatric symptoms are common indicator, with abdominal pain and tachycardia occurring in 90% and 80% of attacks, respectively (Dombeck, T. A. and Satonik, R. C., 2005).

Congenital erythroblastic porphyria (CEP) is an autosomal recessive photosensitive porphyria characterized by an uroporphyrinogen III synthase deficiency. Only about 100-200 cases of the relatively rare disorder has been reported (Sassa and Kappas, 2000). The porphyrin intermediates that accumulate in CEP lead to cutaneous photosensitivity, hemolytic anemia and fragile bones (Sassa and Kappas, 2000).

Porphyria cutanea tarda is the most common porphyria and can be inherited or acquired. Congenital PCT results from decrease uroporphyrinogen III decarboxylase activity and occurs in 1-2 individuals per 100,000. Acquired PCT represents 80-90% of
all cases and among these 80-90% is hepatic with normal erythrocytes. Iron overload is central to the pathogenesis of both acquired and congenital versions. The symptoms can be both neuroviseral and photocutaneous and include other neuropsychiatric conditions, abdominal pain, and chronic blistering lesions of sunlight-exposed skin.

Hereditary coproporphyria is an autosomal dominant acute hepatic porphyria disorder caused by a deficiency in coproporphyrinogen oxidase (Sassa and Kappas, 2000). Like AIP, factors that are associated the induction of heme biosynthesis can induce an attack in which neurological symptoms predominate, although symptoms are usually milder than those displayed with AIP (Dombeck, T. A. and Satonik, R. C., 2005). Mild photosensitivity also occurs in 30% of patients (Dombeck, T. A. and Satonik, R. C., 2005).

Variegate porphyria is autosomal dominant hepatic porphyria and due to a deficiency in protoporphyrinogen oxidase activity (Sassa and Kappas, 2000). It is relatively uncommon except in South Africa where the disorder is prevalent in the white population (Sassa and Kappas, 2000). The clinical symptoms and precipitating factors also resemble those seen with AIP, with the addition of skin photosensitivity symptoms including blisters and superficial ulcers (Dombeck, T. A. and Satonik, R. C., 2005).

Erythropoietic protoporphyria (EPP) is due to a partial deficiency of ferrochelatase activity and is an autosomal dominant disorder (Dombeck, T. A. and Satonik, R. C., 2005). EPP is usually characterized by moderate skin photosensitivity and high concentrations of protoporphyrin IX in the erythrocytes, plasma, and bone marrow (Sassa and Kappas, 2000). Sun exposure is the major contributing factor and symptoms usually occur within minutes of exposure. The phototoxic reactions results in
burns causing intense pain, edema and vesicle formation with repeated insults often leading to “velvet knuckles,” attributed to thick hyperkeratonic skin with deep skin markings over the dorsum the hands (Dombeck, T. A. and Satonik, R. C., 2005).
### Table 1.3: The role of some hemoproteins in biological processes

<table>
<thead>
<tr>
<th>General Roles</th>
<th>Examples</th>
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<tbody>
<tr>
<td><strong>Oxygen carrier</strong></td>
<td>Erythrocyte</td>
</tr>
<tr>
<td></td>
<td>hemoglobin (HbA, HbF, HBA₁, HbA₂)</td>
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<tr>
<td></td>
<td>Cardiac and skeletal muscle</td>
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<tr>
<td></td>
<td>myoglobin</td>
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<tr>
<td></td>
<td>Central and peripheral nervous system</td>
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<td></td>
<td>neuroglobin</td>
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<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>cytoglobin</td>
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<tr>
<td><strong>Electron transport</strong></td>
<td>Mitochondrial electron transport chain</td>
</tr>
<tr>
<td></td>
<td>Cytochrome a, a₁, b, c</td>
</tr>
<tr>
<td><strong>Electron carrier and Oxidation/Reduction</strong></td>
<td>Fatty Acid Metabolism (Fatty acids, Prostaglandins, Eicosanoids)</td>
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<tr>
<td>* (cytochrome P₄₅₀ mediated)</td>
<td>5-lipoxygenase</td>
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<tr>
<td></td>
<td>Metabolism of steroids and related compounds (i.e. vitamin D, bile acids)</td>
</tr>
<tr>
<td></td>
<td>cholesterol 24-hydroxylase</td>
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<td></td>
<td>vitamin D₃ 25-hydroxylase</td>
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<td></td>
<td>retinoic acid hydroxylase</td>
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<td></td>
<td>7-a-hydroxylase</td>
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<tr>
<td><strong>Antioxidant</strong></td>
<td>Drug and xenobiotic oxidation</td>
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<tr>
<td></td>
<td>cytochrome p₄₅₀ mono-oxogenase</td>
</tr>
<tr>
<td><strong>Tryptophan degredation</strong></td>
<td>Reduction of hydrogen peroxide</td>
</tr>
<tr>
<td><strong>Sensors</strong> (NO, CO₂ and CO₂)</td>
<td>catalase peroxidase</td>
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<tr>
<td></td>
<td>glutathione peroxidase</td>
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<tr>
<td></td>
<td>tryptophan pyrrolase</td>
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<tr>
<td></td>
<td><strong>NO sensor</strong></td>
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<tr>
<td></td>
<td>nitric oxide sensitive guanylate cyclase</td>
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<tr>
<td>Classification</td>
<td>Deficient enzyme</td>
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<td>--------------------------------------</td>
<td>---------------------------</td>
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<tr>
<td>Erythropoietic</td>
<td></td>
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<tr>
<td>Congenital erythropoietic porphyria</td>
<td>Uroporphyrinogen III synthase</td>
</tr>
<tr>
<td>Erythropoietic protoporphryia</td>
<td>Ferrochelatase</td>
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<tr>
<td>X-linked dominant protoporphryia</td>
<td>Erythroid aminolevulinate synthase</td>
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<td>Hepatic</td>
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<tr>
<td>ALA dehydratase deficiency porphyria</td>
<td>ALA dehydratase</td>
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<tr>
<td>Acute intermittent porphyria</td>
<td>Porphobilinogen deaminase</td>
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<tr>
<td>Hereditary coproporphyria</td>
<td>Coproporphyrinogen oxidase</td>
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<td>Variegate porphyria</td>
<td>Protoporphyrinogen oxidase</td>
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<tr>
<td>Porphyria cutanea tarda</td>
<td>Uroporphyrinogen decarboxylase</td>
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</tbody>
</table>
The medical significance of other α-oxoamine synthases

The α-oxoamine subfamily of the fold-type I, α-family of PLP-dependent enzymes has 4 members, two of which are involved in the synthesis of protein prosthetic groups which are essential for optimal health, biotin and heme. The two other members of this small family, 2-amino-3-ketoacyl-CoA synthase (KBL) and serine palmitoyltransferase (SPT), are involved in threonine degradation and the synthesis of sphingosine the precursor for spingolipids. While no disease has been associated with KBL, missense mutations in SPT have been associated with hereditary sensory neuropathy I (Bejaoui, Uchida et al., 2002).

SPT catalyzes the decarboxylative condensation reaction of L-serine with palmitoyl-CoA to generate 3-ketodihydrosphingosine which is the first and rate-limiting step in sphingosine biosynthesis. SPT is expressed in many species ranging from bacteria to man. The bacterial enzyme, like most fold-type 1 α-family members, is a water soluble homodimer. However, in mammals SPT is a heterodimer between the SPTLC1 subunit and either SPTLC2 or SPTLC3 and the heterodimer is anchored to the endoplasmic reticulum. Homozygous SPTLC1 and SPTLC2 mice are embryonic lethal (Hojjati, Li et al., 2005). Myriocin, a potent selective SPT inhibitor (Chen, Lane et al., 1999), is an immunosuppressant between 10-100 fold more potent than cyclosporin A (Fujita, Inoue et al., 1994), indicating that sphingolipids have a key role or roles in immunity.

Sphingolipids are a class of lipids derived from the aliphatic amino alcohol sphingosine and include glycosphingolipids and sphingomyelins. These compounds are known to play important roles in signal transduction and cell recognition (Merrill, 2002).
De novo synthesis and turnover of sphingolipids are involved in cell regulation including sphingolipid mediated cell death (Merrill, 2002). Spingolipids are exploited during infection by some bacteria and viruses and have key roles in the toxic action of some exotoxins and endotoxins from a number of microorganisms (Heung, Luberto et al., 2006). Disruption of sphingolipid turnover results in sphingolipidoses, a group of lysosomal storage diseases in which the accumulation of sphingolipids in the central nervous system results in neural degeneration (Jeyakumar, Butters et al., 2002). The roles of this group of lipids are varied and scientists still have much work to do to understand all the mechanisms by which sphingolipids are used to mediate cellular processes.

**Research Aims**

PLP is the active form of vitamin B₆ and is an essential cofactor for enzymes involved a wide array of reactions that are essential for life and the maintenance of health. The small fold-type I subfamily of α-oxoamine synthases, composed of just 4 enzymes, exemplifies this in that they play roles in the biosynthesis of biotin, heme and sphingolipids, biomolecules that are involved in a wide array of cellular processes essential for the maintenance of health. The aim of the research described in the following chapters is to better understand the relationships among this group of enzymes. Understanding the similarities and differences between these enzymes will provide insight into features that provide them with a shared catalytic mechanism, as well as at mechanisms by which these enzymes enforce selectivity.
The studies in the following chapter examine the phylogenetic relationships and the remarkable structural plasticity of the α-oxoamine synthases, as well as the significance of a conserved active site histidine. Chapter 2 explains how sequence and phylogenetic analysis of the α-oxoamine synthases were used to identify amino acids that may be significant in regulating the distinctive substrates specificity of each enzyme. Chapter 3 describes how single chain ALAS dimers and chimeras between ALAS and AONS generated by engineering a single polypeptide chain linking two ALAS polypeptides or the ALAS and AONS polypeptides are used to explore the remarkable structural plasticity exhibited by this group of enzymes. Finally, in chapter 4 the role of an active site histidine that is conserved among the α-oxoamine synthases is explored with the characterization of an ALAS variant in which the histidine is replaced with an alanine.
References


Chapter Two

Sequence and phylogenetic analysis of PLP-dependent α-oxoamine synthases and their role in identifying residues regulating enzyme specificity

Introduction

Pyridoxal 5’-phosphate (PLP) is a versatile cofactor, which exhibits a non-enzymatic ability to catalyze a variety of reactions involving amino acids, including transaminations, racemizations, and α, β eliminations (Snell and Guirard, 1943; Metzler and Snell, 1952; Olivard, Metzler et al., 1952; Longenecker, Ikawa et al., 1957). PLP-dependent enzymes exploit the diverse catalytic repertoire of the PLP-cofactor, as is evident from the fact that this group of enzymes belong to five of six total Enzyme Commission enzyme reaction classes (Mehta and Christen, 2000). PLP-catalyzed reactions utilize the electron withdrawing property of the positively charged heterocyclic nitrogen acting as an “electron sink” which allows for the stabilization of a variety of carbanion intermediates and subsequent labilization of an amino acid α-carbon bond (Christen and Metzler, 1985).

Scrutiny of evolutionary pedigrees in a number of enzyme families has provided significant insight helping to deduce how functional specialization developed. Examination of the evolutionary relationships between the α-family of PLP-dependent enzymes revealed that specialization of the catalytic apparatus for reaction specificity generally requires more extensive adaptations than specialization for a specific substrate.
(Alexander, Sandmeier et al., 1994; Mehta and Christen, 2000; Schneider, Kack et al., 2000). This clear pattern found in the α-family temporal sequence leading to functional specialization prompted the proposal that primitive regio-specific enzymes first diverged into reaction specific protoenzymes, which later acquired substrate specificity and ultimately, phylogenetic diversity (Mehta and Christen, 2000; Christen and Mehta, 2001).

PLP-dependent enzymes are represented by four evolutionary distinct structural groups, with the α-family being the largest and most functionally diverse (Jansonius, 1998; Schneider, Kack et al., 2000; Christen and Mehta, 2001; Eliot and Kirsch, 2004). The CoA-dependent aclytransferases, or α-oxoamine synthases, constitute a small but widespread reaction specific subfamily within the α-family of PLP-dependent enzymes. The α-oxoamine synthase subfamily is comprised of 8-amino-7-oxononanoate synthase (AONS), 5-aminolevulinate synthase (ALAS), serine palmitoyltransferase (SPT), and 2-amino-3-ketobutyrate-CoA ligase (KBL) (Alexeev, Alexeeva et al., 1998; Schneider, Kack et al., 2000; Schmidt, Sivaraman et al., 2001; Astner, Schulze et al., 2005; Yard, Carter et al., 2007), which catalyze Claisen condensations between amino acids and carboxylic acid CoA thioesters (Ferreira, 1999; Bhor, Dev et al., 2006). At the amino acid level, these enzymes share about 12% identity, with the identity between any pair around 30% (Alexeev, Alexeeva et al., 1998). Sequence alignments among members of the α-oxoamine synthase subfamily have been instrumental in the identification of conserved amino acids, while crystallographic and kinetic studies have increased our understanding of the structural and functional significance of these residues. However, the low sequence identities have made it challenging to distinguish residues that differentiate substrate specificity among this group of enzymes.
The recent surge of sequence database submissions resulting from advances in sequencing speed and reliability has generated a reservoir of data that can be used to provide a better understanding of the phylogenetic relationships between the members of the α-oxoamine synthases. A more thorough understanding of the evolutionary progression of α-oxoamine synthases could add insight into the mechanism by which functional specialization developed and provide valuable knowledge for re-engineering substrate specificity.

Because of the diverse catalytic potential of the PLP-cofactor, selectivity of reaction type and substrate specificity also requires PLP-dependent enzymes to significantly diminish or abolish superfluous side reactions and utilization of undesired substrates. Hence, PLP-dependent enzymes commonly demonstrate activity to more than one substrate or reaction (Percudani and Peracchi, 2003). While enzyme promiscuity may seem incompatible with the evolutionary specialization of enzymes, it is often regarded as the origin for the divergent evolution of new specificities and functions (Hult and Berglund, 2007). Engineering substrate promiscuity and/or new substrate specificity in the α-oxoamine synthase family would facilitate a better understanding of the mechanisms by which these enzymes enforce selectivity.

The identification of key residues that distinguish between substrates is a challenge for the protein engineer that also may be overcome using directed evolution. Directed evolution, also known as evolutionary engineering, has emerged as a practical approach because it does not rely on extensive structural information or an understanding of the protein structure, function, or catalytic mechanism (Moore, Jin et al., 1997; Zhao and Arnold, 1997). Directed evolution involves repeated cycles of random mutagenesis
(and/or gene recombination), functional expression, and finally, selection of variants acquiring new features (or exhibiting improvements in the targeted property) (Kuchner and Arnold, 1997). The number of amino acid changes between the parent and the laboratory evolved progeny is significantly smaller than that observed in naturally evolved enzymes. Thus, sequential changes in the in vitro evolved variants may provide insight into residues or positions regulating substrate preference. Incorporating the phylogenetic relationships of both natural and laboratory evolved enzymes with structural data may help identify amino acids key for enzyme selectivity.

AONS and ALAS catalyze decarboxylative condensations leading to the formation of a 2-aminoketone product (Scheme 2.1) (Alexeev, Alexeeva et al., 1998). The sequence, kinetic, and structural similarities between these two enzymes have led to the proposal of a shared kinetic mechanism (Alexeev, Alexeeva et al., 1998; Webster, Campopiano et al., 1998). The relatively short evolutionary space required for developing substrate specificity in the α-family fold–type I PLP-dependent enzymes suggests that alteration of substrate specificity is a practical target for directed evolution. While directed evolution has been used successfully to enhance specificity for minor substrates and relieve product inhibition in other members of the α-family of PLP-dependent enzymes (Yano, Oue et al., 1998; Chow, McElroy et al., 2004; Rothman, Voorhies et al., 2004), there are no records of the use of either approach to redesign α-oxoamine synthases. Reengineering AONS to utilize ALAS substrates would enhance our knowledge of the mechanism by which these enzymes enforce substrate selectivity by identifying residues with key roles in determining substrate specificity.
An enhanced understanding of the phylogenetic relationships among the α-oxoamine synthases is desirable to help identify amino acids that may be relevant for substrate specificity in evolved variants. Therefore, an examination of the relationships among α-oxoamine synthase sequences representing a broad taxonomic and phylogenetic spectrum was performed to help identify residues that may regulate substrate specificity in ALAS, AONS and other α-oxoamine synthases. The reengineering of ALAS to acquire AONS function using directed evolution requires an efficient selection system to identify enzymes exhibiting AONS activity. Thus, one goal of this study is to develop a selection system to identify AONS function in which the growth of biotin-auxotrophic *Escherichia coli* may be rescued by evolved ALAS variants acquiring activity for AONS substrates.

**Experimental Details**

**Sequence search, alignments and phylogenetic analysis**

A series of alignments using AONS, ALAS, KBL, and/or SPT amino acid sequences were performed with MAFFT using mafftE, which incorporates a Ruby script to align input sequences with up to 100 additional homologues from NCBI-BLAST or SwissProt to improve accuracy in the alignment (Katoh, Kuma et al., 2005). Sequences
from the additional homologues aligned using mafftE were selected and added to input sequence sets to obtain a broad phylogenetic distribution of the α-oxoamine synthase family members. To obtain the final sequence set, additional sequences were added to reflect the taxonomic distributions of AONS, ALAS, KBL, and SPT and the final alignments were performed with ClustalW using blssm (blocks amino acid substitution matrices), open gap penalty 10, and gap extension penalty 0.05 (Thompson, Higgins et al., 1994). The ClustalW alignment scores were generated using the SLOW/ACCURATE alignment parameters. The number of amino acid substitutions per site determined from averaging overall sequence pairs is based on the pairwise sequences. Analyses were conducted using the Dayhoff matrix based method in MEGA4 (Schwartz and Dayhoff, 1979; Tamura, Dudley et al., 2007).

The evolutionary history was inferred using the neighbor-joining (NJ), the maximum parsimony (MP), and the minimum evolution (ME) methods (Eck and Dayhoff, 1966; Saitou and Nei, 1987; Rzhetsky and Nei, 1992). Phylogenetic analyses were conducted in MEGA4 (Tamura, Dudley et al., 2007). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 257 positions in the final dataset.

The sum of the length branch in the NJ tree is =16.425 and. The sum of the branch length in the ME tree is 16.330 and the. The ME tree was searched using the close-neighbor-interchange algorithm at a search level of 3 (Tamura, Dudley et al., 2007). The evolutionary distances for the NJ and ME trees were computed using the Poisson correction method. The MP tree was obtained using the close-neighbor-interchange
algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (100 replicates) (Felsenstein, 1985; Nei and Kumar, 2000).

**Construction of ALAS random library**

Random mutagenesis of mature ALAS cDNAs by error-prone PCR was performed under conditions similar to those previously described (Shafikhani, Siegel et al., 1997; Zhao, Giver et al., 1998). In error-prone PCR, the MgCl$_2$ concentration is increased, MnCl$_2$ is added and the dNTP concentration is increased to decrease the fidelity of the Taq polymerase during DNA synthesis (Cadwell and Joyce, 1992). The pGF23 plasmid containing the ALAS cDNA sequence flanked by a 5’ SalI and a 3’ BamHI restrictions site was used as the template (Ferreira and Dailey, 1993). The 5’ primer (TTCTGCTCATGCTCATGTTG) was designed to anneal upstream of the SalI restriction site and the 3’ primer (TACTAGATGTACAGTTCTACGTGCAC) was designed to anneal with the sequence of the vector backbone, downstream of BamHI site. The PCR was performed in 100 μl containing 50 mM KCl, 10 mM Tris (pH 8.3), 7 mM MgCl$_2$, 0.15 mM MnCl$_2$, 0.2 mM dGTP and dATP, 1 mM dCTP and dTTP, 0.1 μM primer and 5 to 10 ng template and 5 units Taq polymerase (Roche, Indianapolis, IN) using a MJ Research MiniCycler. The times and temperatures for annealing, extension, and denaturation were 1 min at 94°C, 1 minute at 50°C and 1 minute at 72°C, respectively. This sequence was repeated for 30 cycles. Subsequently, the PCR product was purified using QIAquick spin columns (QIAGEN, Valencia, CA).

The “megaprimer PCR of whole plasmid”, MEGAWHOP, cloning method was used to subclone the mutant library using the error-prone PCR products as megaprime
(Miyazaki and Takenouchi, 2002). PCR was performed in 100 μl reactions containing 50 mM KCl, 10 mM Tris (pH 8.3), 1 mM MgCl₂ and 2.5 units of Taq polymerase, 0.2 mM each dNTP, 0.5 to 1.0 μg megaprimers and 50-100 ng of pGF23 using an MJ Research MiniCycler. The times and temperatures for annealing, extension, and denaturation were 30 seconds at 94°C, 1 minute at 50°C and 7 minutes at 72°C, respectively. This sequence was repeated for 30 cycles. After the PCR, 10 units of DpnI were added directly to the reaction mixture and incubated at 37°C for 30 minutes. The DpnI digestion was terminated by incubating the reaction at 80°C for 10 minutes. The amplified and DpnI-treated DNA was purified using QiaQuick spin columns (QIAGEN, Valencia, CA).

Competent Escherichia coli DH5α cells were transformed with the Dpn I-treated MEGAWHOP library and grown on Luria-Bertani medium (Bertani, 1951) containing 50 mg/ml ampicillin for 16 hours at 37°C. After incubation, the cells were scraped from the plate and resuspended in sterile buffer P1 (QIAGEN, Valencia, CA). Plasmid DNA from the library of ALAS variants was purified using Q-10 mini columns according to the manufacturers’ protocol (QIAGEN, Valencia, CA).

Selection and bioassay for AONS activity

E. coli strain R872 (Del Campillo-Campbell, Kayajanian et al., 1967), which can only grow on a medium containing AON, biotin or when harboring a plasmid expressing functional AONS, were used to screen for AONS function. Competent R872 E. coli cells were transformed with the variant ALAS plasmid library and plated on M9 minimal medium supplemented with 0.1 % vitamin-free casamino acids and containing 1.5% agar, 0.1 mM CaCl₂, 50 mg/ml ampicillin and 5 to 10 pg/ml biotin. The M9 medium contains
43 mM Na₂HPO₄·7H₂O, 22 mM KH₂PO₄, 8.5 mM NaCl, and 18 mM NH₄Cl, 2 mM MgSO₄, and 0.4% glucose (Smith and Levine, 1964). The plates were incubated at 37°C for 20 hours, followed by 16 hours at room temperature.

The plates were examined for apparent colonies. R872 *E. coli* colonies expressing ALAS variants were selected and used to inoculate 0.3 ml of LB medium and then grown at 37°C while shaking at 115 rpm. Subsequently, 5 ml of M9 minimal medium supplemented with 0.1% vitamin-free casamino acids, 0.1 mM CaCl₂, 50 mg/ml ampicillin and 100 pg/ml biotin was inoculated with 20 μl of the ALAS library expressing-R872 culture. The bacterial cells were incubated for 20 hours at 37°C while shaking at 200 rpm. The cell culture was centrifuged at 4000 x g for 15 minutes and the supernatant was discarded. The cells were washed twice with M-9 minimal medium. To wash the cells, 1.5 ml of M-9 minimal medium was added, the cells were centrifuged at 4000 x g for 15 minutes and the supernatant removed. The washed pellet was resuspended in 150 μl of M-9 minimal medium and 20 μl of the bacterial cell suspension was used to inoculate 1.5 ml of M-9 minimal medium supplemented with 0.1% vitamin-free casamino acid and various biotin concentrations in a 24-well plate. Plates were incubated for 24 hours at 37°C while shaking at 115 rpm and the OD at 600 nm was read at various time intervals between 20 and 50 hours.

**Results**

**Sequence selection and alignment**

A total of 44 amino acid sequences were selected to equitably represent the phylogenetic and taxonomic distribution of each member of the α-oxoamine synthase
subfamily of PLP-dependent enzymes. Hence, 13 AONS, 10 ALAS, 11 KBL, and 10 SPT sequences were selected. The taxon and species of these sequences are given in Table 2.1. Both AONS and SPT sequences were found in four of the 5 kingdoms, with AONS being the most broadly distributed member of the α-oxoamine synthase family.

While all known AONSs, KBLs, ALASs and prokaryotic SPTs are homodimers, eukaryotic SPTs function as heterodimers composed of dissimilar subunits, LCB1 and LCB2, and possess a single PLP-binding moiety, which is attributed to LCB2 (Han, Gable et al., 2006). In this study, SPT1 and SPT2 refer to LCB1 and LCB2 sequences, respectively, and SPT refers to the prokaryotic sequences. Mammals and other vertebrates express genetically distinct housekeeping and erythroid ALAS genes for two distinct isoforms, ALAS1 and ALAS2, respectively (Bishop, 1990; Ferreira and Gong, 1995).

After the removal of the non-homologous terminal residues, the ClustalW aligned sequences ranged from 361 to 409 amino acids. A selected subset of ALAS, KBL and AONS aligned sequences is shown in Figure 2.1 and pairwise alignment scores of all sequences, given in percent identity, are given in Table 2.2. The average evolutionary divergence, deduced by determining the number of amino acid substitutions per site, was 1.5 among all 44 sequences and the AONS sequence set, 0.7 for both the ALAS sequence set and the KBL sequence set, and 0.8 for the SPT2 sequence set.

The sequence alignment of ALAS, AONS, KBL, SPT2 and SPT indicated that nine positions are occupied with completely conserved residues, Gly88, H142, Ser189, Asp214, H217, Lys248, Gly254, Arg356, and Pro364 (Figure 2.1). As inferred from the ALAS and AONS crystal structures (Alexeev, Alexeeva et al., 1998; Webster, Alexeev et
al., 2000; Astner, Schulze et al., 2005), six of the nine conserved residues, Ser189, Asp214, Lys248 and Gly254, correspond to residues interacting with the PLP cofactor. Gly88 and Pro364 appear to bind succinyl-CoA, while Arg356 appears to bind the amino acid carboxylate group, as deduced from the crystal structures of \textit{R. capsulatus} ALAS bound to either glycine or succinyl-CoA substrates (Astner, Schulze et al., 2005).
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<tr>
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FIGURE 2.1  
Alignment of representative sequences of ALAS and KBL with closest AONS sequences. The abbreviations are explained in Table 2.1. Amino acid shading in black and grey represent residues with homology greater than 50% identity or similarity, respectively. Residues in red or blue indicate positions that are conserved (or non-conserved) in ALAS or KBL sequences, respectively. (Numbering used in figure one is based on *R. capsulatus* ALAS crystal structure (Astner, Schulze et al., 2005.)) (Con t on next page.)
Table 4.2

<table>
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**Note:** The table contains data related to various companies, including their names and codes. The specific data values are not clearly visible due to the image resolution.
Phylogenetic analysis of the α-oxoamine synthase family of PLP-dependent enzymes

The neighbor-joining (NJ), maximum parsimony (MP) and minimum evolution (ME) methods were used to infer the evolutionary history of the α-oxoamine synthase family of PLP-dependent enzymes (Eck and Dayhoff, 1966; Saitou and Nei, 1987; Rzhetsky and Nei, 1992; Tamura, Dudley et al., 2007). Analyses were conducted using the MEGA4 software package (Figure 2.2) (Tamura, Dudley et al., 2007). The three methods differ in the way the trees are constructed. Both the NJ and ME are distance matrix methods utilizing pairwise distances for phylogenetic reconstruction. The ME criterion incorporates a tree-search protocol and accepts the tree with the shortest sum of branch lengths, and thus minimizes the total amount of evolution assumed (Rzhetsky and Nei, 1992). The NJ method analyzes only a small number of trees and employs a “greedy search” based on locally optimized choices (Saitou and Nei, 1987). In the MP method, the preferred phylogenetic tree is the tree that requires the least number of evolutionary changes. The MP criterion incorporates a tree search protocol in which trees are scored based on how many evolutionary transitions are required to explain the distribution of each character (Eck and Dayhoff, 1966). The bootstrap method of Felsenstein was used to evaluate the level of confidence associated with the phylogenetic trees; bootstrap values between 70% and 100% indicate significant support for a branch (Felsenstein, 1985; Soltis and Soltis, 2003).

The overall topologies of the three trees have significant congruencies. Each tree contained two main branches, one formed by SPT1s and SPT2s and the second formed by AONSs, ALASs, SPTs and KBLs (Figure 2.2). In all three trees, ALASs were isolated within a single cluster and all KBLs appeared together in a single cluster, which
also included *B. cereus* and *T. kodakarensis* AONSs. The distribution of SPTs and AONSs deviated between the three trees. The bootstrap probability values were generally \( \geq 50\% \) among the branches containing ALASs, KBLs, SPT1s and SPT2s, whereas the branches containing AONSs generally exhibited \( \leq 50\% \) scores. This suggests that larger evolutionary distances separate representative AONS sequences, relative to the evolutionary distance between either ALAS or KBL sequences.
FIGURE 2
Phylogenetic trees for the α-oxoamine synthase family of PLP-dependent enzymes. The phylogenetic trees were derived using the (A) minimum evolution method, (B) maximum parsimony method and (C) neighbor-joining method. Numbers on branches indicate local bootstrap probability with values ≥50% (Felsenstein, 1985). (The branch numbers in the minimum evolution tree represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test. The branch numbers in the maximum parsimony tree represent the percentage of replicate trees in which the associated taxa clustered together, as inferred from 500 replicates. The branch numbers in the neighbor-joining tree represent the confidence probability that the interior branch length is greater than 0 as estimated using the bootstrap test and multiplied by 100.)
Strategy for selection of evolved ALAS variants with AONS activity

The isolation of ALAS variants evolved to acquire AONS activity relied on the development of a biological selection system. The *bioF* mutant *E. coli* R872 cells have no AONS function and thus are biotin-auxotrophic. When grown on a minimal plate, R872 cells cannot grow without biotin supplementation (≥100 pg/ml). Thus the biological system involved the reversion of the biotin auxotrophy by transformation of the *E. coli* R872 cells with ALAS variants exhibiting AONS activity. It should be noted that when transformed with a plasmid expressing *E. coli* AONS, the growth of R872 cells in a medium without biotin was rescued, while transformation of R872 cells with an ALAS expression plasmid did not rescue their growth in a medium without biotin (data not shown).

To increase the probability of selecting ALAS variants which would confer R872 cells the ability to synthesize biotin, the initial round of screening was performed in a medium containing 5 pg/ml biotin, while biotin was omitted in the subsequent rounds of screening to the medium. Thus, a simple and efficient bioassay was developed to further confirm and compare AONS function in ALAS variants selected in the initial screen. The bioassay also took advantage of the *bioF* mutant *E. coli* R872 strain. The assay was based on previous studies by Hwang et. al., who described that the growth of an *E. coli* *bioF* deletion strain in minimal medium was dependent on the addition of biotin, in the 0 - 10 ng/ml concentration range (Hwang, Su et al., 1999). The bioassay was modified to follow growth of R872 cells in 24-well plates (2. 3). Using the modified assay, AONS function in multiple ALAS variants can be efficiently screened by following the growth of R872 cells.
FIGURE 2.3
Growth of R872 cells in minimal media. Growth of bacterial R872 cells as function of biotin concentration. Cell growth was monitoring by determining the absorbance at 600 nm (OD$_{600\text{nm}}$) following 24 hours of incubation in minimal medium or minimal medium supplemented with biotin. (Inset: Linear plot following R872 cell growth with increasing concentrations of biotin.)
Directed Evolution of ALAS and screening for AONS function

Error-prone PCR was used to create an ALAS cDNA library with a low mutation rate and thus reduce the number of simultaneous, hindering amino acid mutations and simplify the analysis of ALAS variants exhibiting AONS function (Zhao, Giver et al., 1998). The library was initially transformed into DH5α E. coli, because high efficiency transformations of the ALAS libraries could not be attained with E. coli R872. Plasmids isolated from approximately 364,000 DH5α transformants were subsequently transformed into R872 cells. Approximately 1.5 x10^6 R872 transformants were plated on minimal plates supplemented with 5 pg/ml biotin to screen for variants exhibiting AONS function. A total of 21 variants were selected for screening using the bioassay.

Of the 21 selected variants screened using the AONS bioassay, one variant (B2) exhibited significantly higher cell density after 24 hours in medium supplemented with 5 and 10 pg/ml of biotin and two (E5 and E6) exhibited increased cell density at a single biotin concentration when compared to R872 cells transformed with an “empty” vector (i.e., containing neither the AONS- nor the ALAS-encoding DNA) (Figure 2.4). To ensure that the increased growth resulted from the transformation with evolved ALAS variants and not reversion of the R872 strain cells, plasmids expressing the ALAS variants were obtained from the cells exhibiting increased growth rates and transformed into new batches of freshly made competent R872 cells. (Plasmid encoding the E6 variant could not be obtained.)

The bioassay was repeated using both the progeny of the screened R872 cells harboring plasmids encoding ALAS variants and R872 cells freshly transformed with the
isolated plasmids. When cell growth was monitored between 20 and 50 hours, growth observed with the freshly transformed cells was comparable to that of bacterial cells transformed with the “empty” vector controls in medium supplemented with 1, 5 or 10 pg/ml of biotin, while the progeny of cells isolated during screening continued to exhibit growth over negative controls at all biotin concentrations (Figure 2.5). This indicated that cells were not rescued by the expression of the ALAS variants selected upon screening for AONS function.
FIGURE 2.4
Growth of R872 cells expressing ALAS variants. Comparison of cell growth as measured by OD at 600 nm for R872 cells expressing ALAS variants and the “empty” vector (Cass3) at 24 hours at 5 (white) and 10 (grey) pg/ml of biotin.
FIGURE 2.5
Comparison of cell growth of freshly transformed R872 cells. R872 cells expressing selected ALAS variants in minimal medium with (A) 10, (B) 5 or (C) 1 pg/ml of biotin. Cell growth was monitored by measuring the OD$_{600}$ nm of the cell cultures. Red lines indicate growth for R872 cells expressing AONS, blue lines indicate growth for cells transformed with the “empty” vector, solid black lines indicate freshly transformed cells and dashed black indicates progeny from initial transformation of selected variants. Symbols indicates ALAS variants expressed in R872 cells, ◆=B2 variant, □=E5 variant and ▲=E3 variant.
The bioassay was repeated using both the progeny of the screened R872 cells harboring plasmids encoding ALAS variants and R872 cells freshly transformed with the isolated plasmids. When cell growth was monitored between 20 and 50 hours, growth observed with the freshly transformed cells was comparable to that of bacterial cells transformed with the “empty” vector controls in medium supplemented with 1, 5, or 10 pg/ml of biotin, while the progeny of cells isolated during screening continued to exhibit growth over negative controls at all biotin concentrations (Figure 2.5). This indicated that cells were not rescued by the expression of the ALAS variants selected upon screening for AONS function.

Identification of residues that potentially regulate $\alpha$-oxoamine synthase specificity

Given that no evolved ALAS variants were generated to identify residues with an ability to influence substrate specificity in the $\alpha$-oxoamine synthase family, we proceeded to scrutinize the primary structure and search for residues potentially involved in the regulation of specificity in ALAS and KBL. Residues were identified that may have a role in controlling specificity by taking advantage of an improved understanding of the evolutionary relationships among this group of enzymes.

The evolutionary space between AONS and ALAS or KBL sequences varied widely, 10-36% or 17-53% identity between AONS and ALAS or AONS and KBL sequences, respectively. Because of this large variation, we reasoned that AONS sequences closest to those of ALAS or KBL were likely to be most similar to a shared ancestor sequence. Therefore, the AONS sequences closest to ALAS and KBL were used to help us suggest residue variations that may have influenced changes in enzyme
specificity. The phylogenetic trees and % identity scores were used to select AONS sequences most similar to ALAS and KBL sequences. AqAe, BaCe, BaSu, and ThKo AONS sequences were most similar to both ALAS and KBL sequence sets. (Table 2.2). (Species abbreviations defined in Table 2.1.)

In order to identify residues that may play a role in determining enzyme specificity, the ClustalW-aligned ALAS and KBL sequences were compared to the 4 selected AONS sequences (Figure 2.1). The 37 residues identified and their structural position relative to the ALAS crystal structure are summarized in Table 2.2. Figures 2.6 and 2.7 illustrate the residues positioned near the SCoA- and glycine-binding pockets, with the majority of the identified residues being located at or adjacent to the active site.

Discussion

Phylogenetic Analysis

The evolutionary distances among all 44 α-oxoamine synthase sequences and the 13 AONS sequences, as determined by the number of amino acid substitutions, is equivalent and twice that observed among ALAS, KBL or SPT2 sequences. Moreover, the clustering of ALASs, KBLs and SPTs in single branches and the bootstrap probability scores below 50% contrast with the relatively broad distribution of AONSs in all three evolutionary trees. Together these data are consistent with the proposition of the appearance of AONS function early in the evolutionary time line of the α-oxoamine synthases and the subsequent development of ALAS, KBL, SPT and SPT1/2 function.

The α-family of PLP-dependent enzymes consists of enzymes essential for amino acid metabolism as well as synthesis of the vital protein cofactors heme and biotin (Ploux
and Marquet, 1992; Ferreira and Gong, 1995). AONS and 7,8 diaminopelargonic acid aminotransferase catalyze the first two common reactions in the biosynthesis of biotin (Schneider, G. and Lindqvist, Y., 2001) and are two of only six members of the $\alpha$-family of PLP dependent enzymes for which sequences have been found in all biological kingdoms (Mehta and Christen, 1998), suggesting that the biotin biosynthesis pathway was likely developed in the universal ancestor cell. Considering that biotin is essential for all organisms and there are no naturally occurring alternative pathways known for biotin biosynthesis, the assertion of an early evolutionary appearance of AONS is plausible.

**Strategy for selection of evolved ALAS variants**

The selection system developed is based on the auxotrophy of the $bioF$ mutant $E. coli$ R872 strain, which does not exhibit AONS function. The R872 strain of $E. coli$ was obtained for this project on the premise that $BioF$ gene, encoding AONS, was knocked out. Late into the first round screening for AONS function, it was discovered that this auxotrophic strain was a mutant and not a knockout strain (Del Campillo-Campbell, Kayajanian et al., 1967).

Utilizing a mutant $BioF$ strain instead of a $BioF$ knockout strain, could permit $BioF$ reversions that restore AONS function and eliminate biotin dependency. Therefore, ALAS variants-encoding plasmids, isolated during the initial screening for AONS function, were retransformed into new batches of R872 cells in order to verify whether the growth observed during selection was not attributed to the reversion of the $BioF$ mutant. Unfortunately, the freshly transformed cells could not grow in a medium supplemented with biotin concentrations used during selection. This almost certainly
indicates that the growth observed during the selection of the ALAS variants resulted from the reversion of the R872 BioF gene and is not attributed to the ALAS variants. This conclusion is further supported by the DNA sequencing data of the expression plasmid for the variant B2, which indicated the absence of amino acid substitutions in the B2 variant-encoding cDNA, albeit the apparent ability of R872 cells harboring the B2 variant to grow in minimal medium or minimal medium supplement with a limited biotin concentration (Figure 2.4).

For directed evolution of AONS function to be feasible, an efficient selection is required. While a biotin auxotroph encoding a mutant BioF gene can effectively select for AONS function, the efficiency is compromised with the screening of large libraries when reversions result in the selection of false positives. The flawed selection strategy prevented the continuation of this project.

Identification of residues that potentially regulate specificity in α-oxoamine synthase

While no ALAS variants with altered substrate specificity were identified using directed evolution, the enhanced understanding of the evolutionary relationships among the α-oxoamine synthases allowed for the identification of residues which potentially influence specificity. The large evolutionary distance among AONS sequences resulting from an early evolutionary appearance of AONS presents the possibility that significant diversity was present in the α-oxoamine synthases prior to the appearance of ALAS and KBL function. Thus, the ALAS and KBL precursor sequences were likely most similar to AONS sequences with the closest identity to the ALAS and KBL sequence sets.

By comparing the ALAS and KBL primary structures with the closest AONS relatives we identified the sequential position of residues that may function to regulate
enzyme specificity (Table 2.3). The ALAS crystal structure from *R. capsulatus* provided insight into the role of various positions identified. The majority of the 37 sequential positions were clustered near the active site (Figure 2.6 and 2.7); in fact, only 4 positions were distal to the active site. Interestingly, nine of the identified active site positions were occupied with residues that interact with either a substrate or the PLP cofactor in the ALAS crystal structure.

In the *R. capsulatus* ALAS crystal structure seven of the identified residues are involved in succinyl-CoA recognition. Gly82, Thr83, Asn85 and Ile86 are located in a glycine-rich stretch directly involved in positioning the carboxylate group of succinyl-CoA (Astner, Schulze et al., 2005). The carboxylate is positioned through a van der Waals interaction with Ile86 and hydrogen bonds between the two carboxylate O and Thr83 and Asn85. Lys156 and Ile149 also have a role in succinyl-CoA recognition through a hydrogen bond between the O3’ of the succinyl-CoA ribose moiety and Lys156-Ne and a hydrophobic interaction between the adenine moiety and Ile149 (Astner, Schulze et al., 2005). Asn362 in the *R. capsulatus* ALAS is equivalent to Arg349 in the *E. coli* AONS crystal structures. Both ALAS Asn362 and AONS Arg349 are hydrogen-bonded to an N-terminal arginine in the holoenzyme (ALAS or AONS) and respective SCoA- or AON-bound structures. This arginine forms a hydrogen bond with the SCoA or AON carboxylate in the ALAS or AONS structure, respectively, and likely has a role coordinating the C-terminal transition upon SCoA or AON binding (Webster, Alexeev et al., 2000; Astner, Schulze et al., 2005).

The crystal structures of α-oxoamine synthases also reveal an active site loop, which upon the transition of the enzyme from the open to closed conformation, closes
over the active site (Webster, Alexeev et al., 2000; Astner, Schulze et al., 2005). In ALAS, the closure of this loop, comprised of residues 358-374, over the active site is important for the proper orientation of succinyl-CoA for catalysis (Astner, Schulze et al., 2005). Three residues identified, Gln359, and Glu379 along with the previously mentioned Asn362, are at positions located on this active site loop in \textit{R. capsulatus} ALAS (Astner, Schulze et al., 2005).

The three above identified residues appear to be involved in positioning the PLP cofactor in the \textit{R. capsulatus} ALAS crystal structure. Tyr116 and Ser277 form two of six hydrogen bonds with the phosphate group (Astner, Schulze et al., 2005). At position 216 of the amino acid alignment, a Val residue is conserved among all ALAS sequences, while Ala is conserved among all AONS, SPT and SPT2 sequences (Figure 2.1). The corresponding residue in the ALAS and AONS crystal structures (Val216 and Ala206, respectively) forms hydrophobic contacts with PLP and functions to stabilize the pyridinium ring from underneath (Alexeev, Alexeeva et al., 1998; Astner, Schulze et al., 2005). Equivalent residues in aspartate aminotransferase and 1-aminocyclopropane-1-carboxylate were mutated to isoleucine, \textit{i.e.}, Ala224Ile and Ile232Ala, respectively, resulting in a 4-10 fold decrease in $k_{cat}/K_m$ and altered pK$_a$ values for the internal aldimine in both enzymes (Eliot and Kirsch, 2002).

Hereditary sideroblastic anemia or XLSA is attributed to point mutations in ALAS2 (Bottomley, May et al., 1995). Five of the residues identified in the \textit{R. capsulatus} ALAS structures are associated with mutations observed in XLSA patients (Astner, Schulze et al., 2005). Mutations at four of these residues, including those previously cited, Lys156 as well as Phy23, Gly273 and Leu133, disrupt the binding of the
succinyl-CoA substrate (Astner, Schulze et al., 2005). XLSA mutations impacting PLP affinity also occur at positions that were identified; these residues are equivalent to Lys30 and the abovementioned Gly273 (Astner, Schulze et al., 2005).

While the significance of the residues occupying the positions identified as determining in substrate selectivity and/or discrimination can not be completely understood with the crystal structures alone, the location of the majority of these residues at or near the active site does not contradict the proposition that they may be important in regulating substrate specificity in ALAS, KBL and AONS. In fact, one third of the positions identified using the alignment are equivalent to ALAS residues that are either involved in direct interactions with succinyl-CoA or PLP, associated with XLSA, or located on an active site loop that functions to position succinyl CoA. This indicates that many of the residues located at the identified positions do have a role in enzyme function and determining function specificity. Furthermore, enzyme-dependent residue variation at each of the positions also suggests that the significance of each position for ALAS, AONS and KBL function is different.

In summary, sequences were selected to represent the broad taxonomic and phylogenetic distribution of each member of the α-oxoamine synthase subfamily of PLP-dependent enzymes and to better comprehend their evolutionary relationships. The enhanced understanding of these relationships may have helped to provide insight into the significance of the primary structure differences in laboratory evolved variants. While no evolved variants were identified, the knowledge gained by the phylogenetic analysis did aid in the identification of sequential positions at which amino acid residues varied according to enzyme function. Furthermore, one third of the identified amino
acids occupied relevant active site positions in the ALAS crystal structure and/or were previously recognized to be mutated in XLSA.
### Table 2.3

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<td>I,E</td>
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<td>N</td>
<td>Adjacent to active site N54 which forms hydrogen bond with the carboxylate of glycine</td>
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<td>A,G</td>
<td>S</td>
<td>Glycine rich stretch that positions terminal carboxylate of SCoA</td>
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<tr>
<td>83</td>
<td>T</td>
<td>S</td>
<td>V</td>
<td>Forms hydrogen bond with SCoA carboxylate O (In AONS, S has key role in governing enzyme specificity&lt;sup&gt;b&lt;/sup&gt;)</td>
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<td>F,T</td>
<td>Glycine rich stretch that positions terminal carboxylate of SCoA</td>
</tr>
<tr>
<td>86</td>
<td>I</td>
<td>V,L,T,A</td>
<td>I</td>
<td>Glycine rich stretch that positions terminal carboxylate of SCoA</td>
</tr>
<tr>
<td>103</td>
<td>L</td>
<td>F,W,L,R</td>
<td>F</td>
<td>Remote</td>
</tr>
<tr>
<td>104</td>
<td>H</td>
<td>X</td>
<td>X</td>
<td>Remote</td>
</tr>
<tr>
<td>106</td>
<td>K</td>
<td>X</td>
<td>X</td>
<td>Remote</td>
</tr>
<tr>
<td>113</td>
<td>S(T)</td>
<td>X</td>
<td>X</td>
<td>Adjacent</td>
</tr>
<tr>
<td>116</td>
<td>Y,F</td>
<td>F,Y</td>
<td>F</td>
<td>Forms hydrogen bond with phosphate O</td>
</tr>
<tr>
<td>117</td>
<td>I,V</td>
<td>X</td>
<td>D(T)</td>
<td>Adjacent</td>
</tr>
<tr>
<td>127</td>
<td>A,G/R</td>
<td>X</td>
<td>L,M</td>
<td>Adjacent to active site and surface loop 130-134</td>
</tr>
<tr>
<td>130-132</td>
<td>M,L,F</td>
<td>-P(N,K)-X</td>
<td>E(N)-D-A/-</td>
<td>Surface loop adjacent to active site</td>
</tr>
<tr>
<td>133-134</td>
<td>C,L/I</td>
<td>-E,I,V</td>
<td>Deleted</td>
<td>Surface loop adjacent to active site (ALAS 133 mutation disrupts SCoA adenine binding pocket reducing SCoA affinity&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td>147</td>
<td>X</td>
<td>D</td>
<td>D</td>
<td>Adjacent to active site</td>
</tr>
<tr>
<td>149</td>
<td>I</td>
<td>V,C,M</td>
<td>V,C,M</td>
<td>Bottom of hydrophobic pocket for SCoA adenine moiety</td>
</tr>
<tr>
<td>156</td>
<td>K</td>
<td>X</td>
<td>K,R</td>
<td>Forms hydrogen bond with SCoA ribose moiety ALAS (ALAS mutation reduces SCoA affinity&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td>159</td>
<td>F,Y</td>
<td>Y</td>
<td>Y(V)</td>
<td>Adjacent to active site</td>
</tr>
<tr>
<td>184</td>
<td>F</td>
<td>T(S)</td>
<td>T</td>
<td>Adjacent to active site</td>
</tr>
<tr>
<td>185</td>
<td>E</td>
<td>D</td>
<td>D</td>
<td>Forms hydrogen bond with H142 which positions PLP from above</td>
</tr>
<tr>
<td>216</td>
<td>V</td>
<td>A</td>
<td>A,C,S</td>
<td>ALAS and AONS supports the PLP ring from below&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>270</td>
<td>Y,F(L,S)</td>
<td>X</td>
<td>K,R</td>
<td>270-272 LOOP between α9 and α10 PLP binding</td>
</tr>
<tr>
<td>271</td>
<td>A(S)</td>
<td>A(M)</td>
<td>S,G</td>
<td>270-272 LOOP between α9 and α10 PLP binding</td>
</tr>
<tr>
<td>272</td>
<td>P,A,S</td>
<td>R(K,T)</td>
<td>R,1</td>
<td>270-272 LOOP between α9 and α10 PLP binding</td>
</tr>
<tr>
<td>273</td>
<td>G(H)</td>
<td>X</td>
<td>P</td>
<td>ALAS mutation disrupts residues involved in both SCoA and PLP binding</td>
</tr>
<tr>
<td>277</td>
<td>T(S)</td>
<td>S</td>
<td>T,Q</td>
<td>Shares hydrogen bond with Phosphate O</td>
</tr>
<tr>
<td>359</td>
<td>Q</td>
<td>Q,L,P</td>
<td>X</td>
<td>Loop of C terminal loop that moves to widen the active site channel in open and closed conformation</td>
</tr>
<tr>
<td>362</td>
<td>N</td>
<td>R(M,E,Y)</td>
<td>V,S,F</td>
<td>Loop of C terminal loop the moves to narrow the active site channel in open and closed conformation</td>
</tr>
<tr>
<td>371</td>
<td>E</td>
<td>X</td>
<td>A(G)</td>
<td>Loop of C terminal loop the moves to narrow the active site channel in open and closed conformation</td>
</tr>
<tr>
<td>379</td>
<td>P</td>
<td>A,S,L</td>
<td>A</td>
<td>Remote-Possible role in intersubunit interaction</td>
</tr>
</tbody>
</table>

X = none conserved  
<sup>a</sup>Italic = Occurs in single sequence

<sup>a</sup>(Astner, Schulze et al., 2005)<sup>b</sup>(Alexeev, Alexeeva et al., 1998)
FIGURE 2.6
Succinyl-CoA binding pocket of *R. capsulatus* ALAS. Ribbon structure of the ALAS homodimer (PDB code: 2BWO) active site in which one subunit is depicted in blue with the active site loop in green and the adjacent subunit is in grey. (A) and (B) Cartoon representation of active site residues with the succinyl-CoA substrate (red) depicted in ball and stick representation (carbon in green, oxygen in red, nitrogen in blue). Variable residues according to enzyme function within the α-oxoamine synthase family, which interact with or lie adjacent to succinyl-CoA, are in yellow and residues that lie adjacent to active site loop are green. Images were generated with Deep View (Guex and Peitsch, 1997; Kaplan and Littlejohn, 2001).

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FIGURE 2.7
Active site interactions with PLP cofactor and glycine substrate in *R. capsulatus* ALAS
(A) Ribbon structure of the ALAS homodimer active site (PDB code: 2BWP) in which one subunit is depicted in blue and the adjacent subunit is in grey. Cartoon representation of active site residues with the PLP-bound glycine substrate (red) depicted in ball and stick representation (carbon in green, oxygen in red, nitrogen in blue). Variable residues according to enzyme function within the α-oxoamine synthase family, which interact with or lie adjacent to PLP or glycine, are in green. (B) Ball and stick representation with ribbon structure removed for clarity. (Guex and Peitsch, 1997; Kaplan and Littlejohn, 2001)
References


Thompson, J. D., D. G. Higgins, et al. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,


Chapter Three

Functional asymmetry for active sites of single chain homo- and chimeric dimers of 5-aminolevulinate synthase and 8-amino-7-oxononanoate synthase

Abstract

5-Aminolevulinate synthase (ALAS) and 8-amino-7-oxononanoate synthase (AONS) are homodimeric members of the α-oxoamine synthase family of pyridoxal 5’-phosphate (PLP)-dependent enzymes. Previously, linking two ALAS subunits into a single polypeptide chain dimer resulted in an enzyme (ALAS/ALAS) with a significantly greater turnover number than that of wild-type ALAS. To examine the contribution of each active site to the enzymatic activity of ALAS/ALAS, the conserved lysine, which covalently binds the PLP cofactor, was substituted with alanine yielding single chain dimeric ALAS variants with one of the two active sites having no measurable enzymatic activity. Albeit the amount of ALA produced during the first turnover was identical in both active sites of ALAS/ALAS, the $k_{\text{cat}}$ values of the variants varied significantly (4.4 ± 0.2 min$^{-1}$ vs. 21.6 ± 0.7 min$^{-1}$) depending on which active site harbored the mutation. Chimeric ALAS/AONS proteins exhibited a distinct oligomeric state from that of ALAS/ALAS, with two sites having ALAS activity and two AONS activity. Remarkably, while the steady-state rates for the ALAS and AONS activities were lower than in the parent enzymes, the reactivity of the ALAS sites in ALAS/AONS was similar to that of wild-type ALAS. We propose that the different contribution of each active site
to the steady-state activity of ALAS/ALAS and the reduced steady-state activities of the
ALAS/AONS chimera, compared to the parent enzymes, relate to different extents of
protein conformational changes associated with product release due to the strain caused
with the linking the two ALAS (or ALAS and AONS) subunits.

Introduction

Pyridoxal 5’-phosphate (PLP) is a necessary cofactor for a catalytically versatile
class of enzymes (Ferreira and Gong, 1995). PLP-dependent enzymes that catalyze
reactions involving amino acids share the common mechanism of using the electron
withdrawing properties of the PLP cofactor to labilize bonds to the substrate α-carbon
(Christen and Mehta, 2001).

PLP-dependent enzymes have been classified according to fold types inferred
from three-dimensional structures and reaction specificity (Christen and Mehta, 2001;
Eliot and Kirsch, 2004). The largest and most diverse group is the aspartate
aminotransferase superfamily or fold-type I, α–family (Paiardini, Bossa et al., 2004).
The α-oxoamine synthases constitute a small but widespread subfamily within fold-type I
PLP-dependent enzymes, comprising 8-amino-7-oxononanoate synthase (AONS), 5-
aminolevulinate synthase (ALAS), serine palmitoyltransferase (SPT), and 2-amino-3-
oxobutyrate CoA ligase (Alexeev, Alexeeva et al., 1998; Schneider, Kack et al., 2000;
Schmidt, Sivaraman et al., 2001; Astner, Schulze et al., 2005; Yard, Carter et al., 2007).
These four members of the α-oxoamine family share about 12% identity at the amino
acid level, while the amino acid sequence identity between any pair is around 30%
(Alexeev, Alexeeva et al., 1998). The three-dimensional structures for all four α-
oxoamine synthases are highly conserved, as demonstrated by the Cα root-mean-square deviation values for SPT of 1.4-1.6 Å relative to the other three members (Yard, Carter et al., 2007).

AONS and ALAS catalyze Claisen condensations between amino acids and acyl-CoA thioesters with concomitant decarboxylation of the amino acid leading to the formation of a 2-aminoketone product, CoA and carbon dioxide (Ferreira and Gong, 1995; Alexeev, Alexeeva et al., 1998). AONS catalyzes the reaction between alanine and pimeloyl-CoA to give 8-amino-7-oxononanoate (AON), whereas ALAS catalyzes the reaction between glycine and succinyl-CoA to give 5-aminolevulinic acid (ALA) (Scheme 3.1). Both AON and ALA are essential metabolic compounds: the first is an intermediate in biotin synthesis (Ploux and Marquet, 1992) and the second is an intermediate in the tetrapyrrole biosynthetic pathway (Ferreira and Gong, 1995; Tan and Ferreira, 1996).

\[
\begin{align*}
\text{HO-}\text{R-}\text{NH}_2 + \text{R'}-\text{SCoA} & \rightarrow \text{R'}-\text{R-}\text{NH}_2 + \text{CoASH} + \text{CO}_2 \\
R &= \text{II and } R' = \text{CII}_2\text{CO}_2^- \text{ for ALAS} \\
R &= \text{CH}_3 \text{ and } R' = (\text{CH}_2)_4\text{CO}_2^- \text{ for AONS}
\end{align*}
\]

**SCHEME 3.1 Reaction catalyzed by ALAS and AONS**

ALAS and AONS function as homodimers (Tan and Ferreira, 1996; Alexeev, Alexeeva et al., 1998; Astner, Schulze et al., 2005). The active site is located at the
subunit interface, where the PLP cofactor is covalently bound to an active site lysine through a Schiff base linkage (Ferreira, Neame et al., 1993; Alexeev, Alexeeva et al., 1998; Astner, Schulze et al., 2005). Each monomer consists of three domains, a short N-terminal domain (~50 residues), a central catalytic core (~250 residues), and a C-terminal domain (~100 residues). Although all three domains participate in dimerization, the catalytic domain contributes the most to the dimeric interface (Alexeev, Alexeeva et al., 1998; Astner, Schulze et al., 2005; Yard, Carter et al., 2007).

Despite the highly conserved tertiary structure of the α-oxoamine synthases, the plasticity of the PLP-binding and active site is quite remarkable, as demonstrated with circularly permuted murine erythroid ALAS variants (Cheltsov, Barber et al., 2001; Cheltsov, Guida et al., 2003). Circular permutation of ALAS, which changed the primary sequence without altering the amino acid composition, did not prevent folding of the polypeptide chain into a structure compatible with binding of the PLP cofactor and assembly of the two subunits into a functional enzyme (Cheltsov, Barber et al., 2001; Cheltsov, Guida et al., 2003). In fact, the circularly permuted ALAS variants were able to form tertiary structures similar to wild-type (WT) ALAS that retained enzymatic function in spite of the rearrangement of the secondary structural elements (Cheltsov, Barber et al., 2001). Cheltsov et al. (Ferreira and Cheltsov, 2002) argued that a PLP fold and enzymatic activity are achievable as long as the polypeptide chain of ALAS (and presumably of other α-oxoamine synthases) folds to allow binding of the cofactor and correct positioning of the catalytic residues.

Structural plasticity in the α-oxoamine synthase family has also been substantiated with the creation of a “single chain dimeric” ALAS (Cheltsov, Barber et al.,
2001; Cheltsov, Guida et al., 2003; Zhang, Cheltsov et al., 2005), the discovery of a marine viral single-chain SPT and construction of yeast and mammalian single-chain SPT chimeras (Han, Gable et al., 2006). The “single chain, dimeric” ALAS (i.e., ALAS/ALAS), created by linking two ALAS polypeptide chains, exhibited distinct spectroscopic properties and substantially greater enzymatic activity than WT ALAS (Zhang, Cheltsov et al., 2005). Single-chain chimeras formed from either the two yeast or mammalian SPT subunits, LCB1 and LCB2, displayed novel substrate specificity (Han, Gable et al., 2006). As a matter of fact, similarly to the marine viral single-chain SPT and in contrast to the naturally occurring eukaryotic, heterodimeric SPTs, the yeast and mammalian single-chain SPT chimeras prefer myristoyl-CoA rather than palmitoyl-CoA as substrate (Han, Gable et al., 2006). Essentially, these findings indicate that significant flexibility is present in the architecture and formation of the PLP-binding and active sites of α-oxoamine synthases.

To address whether 1) each of the two active sites in the “single chain dimeric” ALAS contributes equally to the overall enzymatic activity and 2) the structural plasticity and flexibility in active site formation can be extended to chimeras between different members of the α-oxoamine synthase family, we characterized “single chain dimeric” ALAS variants, in which one of the two active sites had no measurable enzymatic activity, and single-chain chimeras between WT or mutated ALAS and AONS. We report that each of the two active sites in ALAS/ALAS contributes differently to the steady-state activity of the enzyme, even though the amount of ALA produced during the first turnover is identical in both active sites. Further, while the chimeric ALAS/AONS proteins tolerate a high degree of structural plasticity, the oligomeric state and the active
site arrangement of the chimeric proteins differ from those of proteins generated from the fusion of identical polypeptide chains (e.g., ALAS/ALAS).
Material and Methods

Materials

The following reagents were purchased from Sigma-Aldrich Chemical Company: DEAE-Sephacel, Ultrogel AcA-44, β-mercaptoethanol, PLP, succinyl-CoA, ALA-hydrochloride, α-ketoglutaric acid, α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, HEPES-free acid, MOPS, tricine, thiamin pyrophosphate, NAD⁺, pyruvic acid, the gel filtration molecular weight markers kit (cytochrome c, carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase, β-amylase), and the bicinechonic acid protein determination kit. Glycerol, alanine, glycine, disodium ethylenediamine tetraacetic acid dihydrate, ammonium sulfate, magnesium chloride hexahydrate, perchloric acid, and potassium hydroxide were acquired from Fisher Scientific. Sodium dodecyl sulfate polyacrylamide gel electrophoresis reagents were acquired from Bio-Rad. All restriction enzymes, Vent DNA polymerase, and T4 DNA ligase were from New England Biolabs. Superdex 200 gel filtration resin was from Amersham Biosciences-GE Healthcare and DNA oligonucleotides were from Integrated DNA Technologies. Escherichia coli R872 strain and pET6HAONS were generous gifts from Dr. Dominic Campopiano (University of Edinburg). E. coli HU227 strain was a generous gift from Dr. Charlotte S. Russell (City University of New York).

Construction of ALAS/ALAS and ALAS/AONS chimeric expression plasmids

Chimeras between murine, mature erythroid ALAS and E. coli AONS or between mutated forms of either ALAS or AONS and the WT enzymes were engineered using pGF23 as the expression vector (Figure 3.1, Table 3.1). The pGF23 plasmid contains the
sequence for murine, mature erythroid ALAS under the control of the alkaline phosphatase promoter (Ferreira and Dailey, 1993). pTDT1, an expression plasmid for 6x-histidine-tagged *E. coli* AONS, was constructed by PCR-amplification of the *E. coli* *bioF* gene, which encodes the AONS protein, and subcloning of the PCR product into pGF23, such that the *bioF* gene replaced the ALAS-encoding fragment (see Supporting Information for Experimental Details).

The pTDT5 and pTDT4 expression plasmids (Table 3.1) were constructed to yield chimeric proteins between ALAS and AONS (Figure 3.1). Using the 5’ to 3’ convention for the “chimeric gene” under the control of the alkaline phosphatase promoter, pTDT5 contains the cDNA coding for ALAS linked to the following *bioF* gene for AONS through a *Mfe*I site, whereas in pTDT4, the *bioF* gene precedes the ALAS cDNA (Figure 3.1). These constructs were based on the pAC1 plasmid (Cheltsov, Barber et al., 2001; Cheltsov, Guida et al., 2003; Zhang, Cheltsov et al., 2005) (Figure 3.1), which contains two tandem ALAS cDNA sequences separated by an *Mfe*I cloning site (see Supporting Information for Experimental Details).

The pTDT12 and pTDT17 expression plasmids (Table 3.1) were constructed using the pGF27 expression plasmid for the ALAS$^{K313A}$ variant (Ferreira, Vajapey et al., 1995) as the starting material for a DNA piece coding for the ALAS$^{K313A}$ mutation. The pGF27 plasmid was digested with *Kpn*I and *Xba*I and the ALAS$^{K313A}$-encoding fragment was ligated into pTDT4 and pTDT5 digested with the same enzymes.

The pTDT8 plasmid (Table 3.1) was designed to encode a full-length ALAS with phenylalanine-341 mutated to alanine. To introduce the F341A encoding mutation into
ALAS cDNA, the method previously described by Gong et al. was followed (Gong, Hunter et al., 1998) (see Supporting Information for Experimental Details).

The pTDT14 and pTDT15 expression plasmids (Table 3.1) were constructed using pTDT8 as the source plasmid for the ALAS$^{F341A}$-encoding fragment. The pTDT8 plasmid was digested with Kpn I and Xba I and the fragment containing the ALAS$^{F341A}$ mutation was isolated and subcloned into pTDT5 and pTDT4 to generate pTDT14 and pTDT15, respectively.

The pTDT7 expression plasmid encodes the ALAS/AONS chimera in which the AONS active-site lysine involved in the Schiff base linkage with the PLP cofactor, K236, is mutated to an alanine (Table 3.1; Figure 3.1). The method described by Gong et al. (11) was used to introduce the K236A-encoding mutation in the bioF gene harbored in pTDT5 (see Supporting Information for Experimental Details).

The pCA1 and pMG1 expression plasmids, encoding ALAS$^{K313A}$/ALAS and ALAS/ALAS$^{K313A}$, respectively, were constructed using the pAC1 and pGF27 plasmids (Table 3.1; Figure 3.1) (see Supporting Information for Experimental Details).
Table 3.1: Nomenclature defining the plasmids and enzymes described in this report

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Protein ²</th>
<th>Description</th>
<th>Mass ³</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGF23</td>
<td>ALAS</td>
<td>ALAS homodimer</td>
<td>56 kD</td>
<td>(Ferreira and Dailey, 1993)</td>
</tr>
<tr>
<td>pTDT1</td>
<td>AONS</td>
<td>AONS homodimer</td>
<td>40 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pGF27</td>
<td>ALAS ³¹³A</td>
<td>ALAS homodimer harboring the K313A mutation</td>
<td>56 kD</td>
<td>(Ferreira, Vajapey et al., 1995; Hunter and Ferreira, 1999)</td>
</tr>
<tr>
<td>pTDT8</td>
<td>ALAS ³⁴¹A</td>
<td>ALAS homodimer harboring the F341A mutation</td>
<td>56 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pAC1</td>
<td>ALAS/ALAS</td>
<td>Single polypeptide ALAS homodimer</td>
<td>112 kD</td>
<td>(Zhang, Cheltsov et al., 2005)</td>
</tr>
<tr>
<td>pCA1</td>
<td>ALAS ³¹³A /ALAS</td>
<td>Chimera of ALAS ³¹³A and ALAS</td>
<td>112 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pMG1</td>
<td>ALAS/ALAS ³¹³A</td>
<td>Chimera of ALAS and ALAS ³¹³A</td>
<td>112 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pTDT5</td>
<td>ALAS/AONS</td>
<td>Chimera of ALAS and AONS</td>
<td>96 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pTDT4</td>
<td>AONS/ALAS</td>
<td>Chimera of AONS and ALAS</td>
<td>96 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pTDT12</td>
<td>ALAS ³¹³A /AONS</td>
<td>Chimera of ALAS ³¹³A and AONS</td>
<td>96 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pTDT17</td>
<td>AONS/ALAS ³¹³A</td>
<td>Chimera of AONS and ALAS ³¹³A</td>
<td>96 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pTDT14</td>
<td>ALAS ³⁴¹A /AONS</td>
<td>Chimera of ALAS ³⁴¹A and AONS</td>
<td>96 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pTDT15</td>
<td>AONS/ALAS ³⁴¹A</td>
<td>Chimera of AONS and ALAS ³⁴¹A</td>
<td>96 kD</td>
<td>This study</td>
</tr>
<tr>
<td>pTDT7</td>
<td>ALAS /AONS ²³⁶A</td>
<td>Chimera of ALAS and AONS ²³⁶A</td>
<td>96 kD</td>
<td>This study</td>
</tr>
</tbody>
</table>

²In the linked proteins the first abbreviation refers to the N-terminal enzyme. ³Monomeric molecular
FIGURE 3.1
Ribbon representation ALAS and AONS homodimers and schematic representation of expression plasmids (A-B) ALAS and AONS homodimers in ribbon representation with one subunit shown in yellow and the central catalytic core, N-terminal domain, and C-terminal domain of the second subunit rendered in dark, medium and light blue, respectively. (A) ALAS homodimer from *R. capsulatus* (PDB code: 2BWN). The PLP cofactor (red), the active site lysine (green) involved in the Schiff base linkage with PLP (K248 in *R. capsulatus* ALAS and K313 in murine erythroid ALAS) and F276 (purple; F341 in murine erythroid ALAS) are depicted in ball-and-stick representation. (B) AONS homodimer from *E. coli* (PDB code: 1BS0). The PLP cofactor (red) and the active site lysine (green) involved in the Schiff base linkage with PLP (K236) are depicted in ball-and-stick representation. (C) Schematic representation of the expression plasmids for mutated ALAS, AONS, ALAS/ALAS variants, and single-chain chimeras between, ALAS (WT or mutated) and AONS (WT or mutated). Each of the expression plasmids contains a DNA fragment, encoding either a single protein or a chimeric protein, under the control of the *phoA* promoter (Ferreira and Dailey, 1993) and possesses an ampicillin resistance selectable marker. (See “Experimental Procedures” for details). *Amp′*, ampicillin resistance gene; *Bam HI, Mfe I* and *Sal I*, cloning sites.
Biological screening for ALAS and AONS function.

*HemA* *E. coli* strain HU227 can only grow in a medium containing ALA, hemin or when harboring an expression plasmid for ALAS or a functional ALAS variant (Sasarman, Surdeanu et al., 1968; Li, Brathwaite et al., 1989). *BioF* *E. coli* R872 strain can only grow in a medium containing AON, biotin or when harboring an expression plasmid for AONS or a functional AONS variant (Del Campillo-Campbell, Kayajanian et al., 1967). Competent *E. coli* HU227 and R872 cells were transformed by electroporation with expression plasmids containing the ALAS and AONS chimeric constructs. To screen for ALAS function, transformed HU227 cells were plated on Luria-Bertani medium (0.5% yeast extract, 1% tryptone, 1.0% NaCl and 1.5% agar) containing 50 mg/ml ampicillin. To screen for AONS function, transformed R872 cells were plated on M9 minimal medium containing 50 mg/ml ampicillin. M9 medium contains 1X M9-salts (12.8 g Na₂HPO₄-7H₂O, 3.0 g KH₂PO₄, 0.5g NaCl, and 1.0g NH₄Cl per 1 L ), 2 mM MgSO₄, 0.1 mM CaCl₂, 0.4 % glucose, 0.1 % vitamin-free casamino acids and 1.5% agar.

Purification of ALAS, AONS, ALAS/ALAS, ALASK₃₁₃₄/ALAS, ALAS/ALASK₃₁₃₄ and ALAS/AONS.

The purification of ALAS from *E. coli* DH5α cells harboring pGF23 was as described by Ferreira and Dailey (Ferreira and Dailey, 1993). Recombinant *E. coli* AONS and chimeric ALAS/AONS were purified from *E. coli* DH5α cells harboring pTDT1 and pTDT5, respectively. Tandem ALAS variants (*i.e.*, ALAS/ALAS, ALASK₃₁₃₄/ALAS and ALAS/ALASK₃₁₃₄) were purified from *E. coli* strain BL21(DE3) overproducing cells harboring pAC1, pCA1 or pMG1. *E. coli* cells harboring any of the six expression
plasmids were grown in MOPS medium containing ampicillin and harvested as previously described (Ferreira and Dailey, 1993). The cell pellet was resuspended in buffer A (20 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol, 1 mM EDTA, 20 mM PLP and 5 mM β-mercaptoethanol) with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptine, 1 μg/ml pepstatin and 1 mg/ml PMSF) as previously described (Ferreira and Dailey, 1993). The steps following cell lysis and centrifugation were essentially as described in (Ferreira and Dailey, 1993) with slight modifications. Specifically, for both AONS and ALAS/AONS, the initial ammonium sulfate fractionation step was 20%. After stirring for 20 min at 4 °C, the solution was centrifuged at 27,000xg for 30 min at 4 °C, and the supernatant was further fractionated with ammonium sulfate to a final concentration of 40%.

For the purification of AONS, the protein pellet was resuspended in buffer A and loaded onto an Ultrogel ACA-44 gel filtration column equilibrated with buffer A. The protein solution was adjusted to 20% (w/v) ammonium sulfate and the subsequent chromatographic steps using Phenyl-Sepharose and Q-Sepharose anion exchange columns were as previously described (Webster, Alexeev et al., 2000).

The purification of ALAS/ALAS from bacterial cells harboring the pAC1 expression plasmid (Table 3.1) was according to a previously published method (Cheltsov, Barber et al., 2001; Cheltsov, Guida et al., 2003; Zhang, Cheltsov et al., 2005). For the tandem ALAS variants, ALASK313A/ALAS and ALASK313A/ALAS encoded by pCA1 and pMG1, respectively (Table 3.1), the protein pellet was resuspended in buffer A and loaded onto a Superdex 200 column equilibrated with buffer A. The fractions containing protein were pooled and loaded onto a DEAE-sephacel resin equilibrated with
buffer A. The resin was washed with buffer A, and the protein was eluted with buffer A containing 70 mM KCl. For the purification of ALAS/AONS, the protein pellet obtained after 40% ammonium sulfate fractionation was resuspended in buffer A, pH 7.9, and loaded onto an Ultrogel ACA-44 column equilibrated with the same buffer. The fractions containing protein were pooled and loaded onto a Q-Sepharose column equilibrated with buffer A, pH 7.9. The Q-Sepharose resin was washed with buffer A, pH 7.9, containing 25 mM KCl, until Abs$_{280}$ of the “washed proteins” was lower than 0.1; ALAS/AONS was eluted from the Q-Sepharose resin with a 100 mM to 150 mM KCl gradient in buffer A, pH 7.5. The protein purity was assessed using SDS-PAGE. When the final product did not meet our purity criteria (i.e., over 95% homogeneity as judged by SDS-PAGE), size exclusion chromatography with Sephadex 200 resin was used to eliminate protein contaminants. Protein-containing fractions were pooled and concentrated in an Amicon 8050 stirred cell with an YM30 membrane. The purified and concentrated enzyme (WT ALAS, AONS, ALAS/ALAS, ALAS$^{K313A}$/ALAS, ALAS/ALAS$^{K313A}$ or ALAS/AONS) was stored in liquid nitrogen.

Protein concentrations were determined with the bicinchoninic acid method using bovine serum albumin as the standard (Smith, Krohn et al., 1985). ALAS/AONS concentrations are reported on the basis of a subunit molecular mass of 96 kD, while ALAS/ALAS$^{K313A}$ and ALAS$^{K313A}$/ALAS concentrations are reported on the basis of a subunit molecular mass of 112 kD. (ALAS and AONS have monomeric molecular masses of 56 kD and 40 kD, respectively (Ferreira and Dailey, 1993; Alexeev, Alexeeva et al., 1998)).
Molecular mass determination by gel filtration chromatography.

The native molecular mass of ALAS/AONS was determined using gel filtration chromatography as previously described by Cheltsov et al. (Cheltsov, Barber et al., 2001).

Pimeloyl-CoA synthesis.

Pimeloyl-CoA was synthesized as previously described by Ploux and Marquet (Ploux and Marquet, 1992). Purity was assessed by reverse-phase HPLC and concentration was determined by measuring the absorbance at 260 nm and using an $\varepsilon_{260\text{nm}} = 16800 \text{ M}^{-1}\text{cm}^{-1}$.

Spectroscopic measurements.

Absorption spectra were acquired at ambient temperature using a Shimadzu UV 2100 dual beam spectrophotometer, with a reference containing all components except the purified enzyme. Fluorescence spectra were collected on a Shimadzu RE-5301 PC spectrofluorophotometer using protein concentrations of 2-4 μM. Fluorescence blank spectra were collected from samples containing all components except enzyme immediately prior to the measurement of samples containing enzyme. The blank spectra were subtracted from the spectra of samples containing enzyme.

Steady-state kinetic characterization of ALAS/AONS, ALAS$^{K313A}$/ALAS, and ALAS/ALAS$^{K313A}$.

ALAS steady-state activity of the ALAS/AONS chimera, ALAS$^{K313A}$/ALAS and ALAS/ALAS$^{K313A}$ was determined at 30°C using a continuous spectrophotometric assay as described previously for WT ALAS (Hunter and Ferreira, 1995). AONS steady-state activity of the ALAS/AONS chimera was determined at 30°C using a coupled
enzymatic spectrophotometric assay for AONS (Webster, Campopiano et al., 1998), which is similar to that developed for determination of ALAS activity (Hunter and Ferreira, 1995). Briefly, in the latter assay, \( \alpha \)-ketoglutarate dehydrogenase was replaced by pyruvate dehydrogenase as the coupling enzyme, and the reactions contained 20 mM HEPES, pH 7.5, 3 mM MgCl\(_2\), 1 mM pyruvic acid, 1 mM NAD\(^+\) and 0.25 to 1 \( \mu \)M enzyme. Data were acquired using a Shimadzu UV 2100 dual-beam spectrophotometer. Enzymatic activity data were plotted vs. substrate concentration in which one of the substrate concentrations varied, while the second was kept constant. The steady-state kinetic parameters (i.e., \( K_{m}^{\text{Gly}} \), \( K_{m}^{\text{SCoA}} \), and \( k_{\text{cat}} \) of the ALAS/AONS chimera, \( \text{ALAS}^{\text{K313A}}/\text{ALAS} \) and \( \text{ALAS}/\text{ALAS}^{\text{K313A}} \) and \( K_{m}^{\text{Alanine}} \), \( K_{m}^{\text{PCoA}} \), and \( k_{\text{cat}} \) of the ALAS/AONS chimera) were determined by fitting the data to the Michaelis-Menten equation using non-linear regression analysis software.

Rapid chemical quenched-flow experiments and data analysis.

Rapid chemical quenched-flow experiments were performed using a SFM-400/Q mode quenched-flow apparatus (BioLogic Science Instruments, France), equipped with a circulating water bath to control the temperature of the reactants as described in Zhang and Ferreira (Zhang and Ferreira, 2002). ALA concentration in the quenched samples was also determined as previously described (Zhang and Ferreira, 2002). ALA produced at different reaction times were plotted against time and fitted to equation 1 (Yard, Carter et al., 2007), using the nonlinear least-squares regression analysis program SigmaPlot, where \( P \) represents the product concentration at an aging time \( t \), \( A \) is the amplitude of the
burst phase, \( k_b \) is the burst rate constant, \( k_a \) is the steady-state rate constant, and \( E_0 \) is the total enzyme concentration (Zhang and Ferreira, 2002).

\[
P_t = A(1 - e^{-k_b t}) + k_a E_0 t \quad \text{Equation 1}
\]

**Results**

*In vivo activity screen of ALAS\(^{K313A}/\text{ALAS and ALAS}/\text{ALAS}^{K313A}$.*

To rapidly verify if ALAS\(^{K313A}/\text{ALAS and ALAS}/\text{ALAS}^{K313A}$, which harbor the K313A mutation in either of the two linked ALAS subunits, exhibited ALAS activity, positive genetic complementation of hemA$^-\text{E. coli}$ HU227 cells was employed. Since HU227 cells can only grow in a medium supplemented with ALA (Li, Brathwaite et al., 1989) or when transformed with functional ALAS expression plasmids (Gong and Ferreira, 1995; Tan and Ferreira, 1996) the rescue of HU227 cells in a non-ALA supplemented medium indicates the production of a variant with ALAS activity. Indeed, while HU227 cells harboring the ALAS homodimer or the single chain dimeric ALAS/ALAS can grow in a medium without ALA (Gong and Ferreira, 1995; Tan and Ferreira, 1996), HU227 cells overproducing the K313A homodimer cannot (Tan and Ferreira, 1996) (Table 3.2). Both ALAS\(^{K313A}/\text{ALAS and ALAS}/\text{ALAS}^{K313A}$ variants retained function as indicated by the ability of transformed HU227 cells to grow when harboring either variant (Table 3.2).
Spectroscopic characterization of $\text{ALAS}^{K313A}/\text{ALAS}$ and $\text{ALAS}/\text{ALAS}^{K313A}$.

Although, as previously reported (Zhang, Cheltsov et al., 2005), the UV/visible absorbance spectra for both ALAS and the single chain dimeric ALAS/ALAS exhibited the characteristic maxima for PLP-dependent enzymes at $\sim330$ and $\sim420$ nm, the prominence of these absorbance bands varied between the spectra of the two proteins (Figure 3.2). The absorbance band at $\sim420$ nm was more prominent in the spectrum of ALAS/ALAS than that of ALAS, whereas the absorbance band at $330$ nm was less distinct in ALAS/ALAS. Indeed, at pH 7.5, the ratio of the 420 nm to 330 nm absorbance changed from 0.44 for ALAS to 0.67 for ALAS/ALAS (Figure 3.2). Similarly, the UV/visible absorbance spectra of the $\text{ALAS}^{K313A}/\text{ALAS}$ and $\text{ALAS}/\text{ALAS}^{K313A}$ variants retained the characteristic maxima at $\sim330$ and $\sim420$ nm while the prominence of the two absorbance bands varied, resulting in a 420 to 330 nm absorption ratio of 0.35 and 0.58 at pH 7.5 for $\text{ALAS}^{K313A}/\text{ALAS}$ and $\text{ALAS}/\text{ALAS}^{K313A}$, respectively. The $\sim330$ and $\sim420$ nm maxima were previously attributed to the substituted aldimine and ketoenamine forms of the internal aldimine between PLP and the $\varepsilon$-amino group of K313, respectively (Zhang, Cheltsov et al., 2005). Clearly, the mutation of the lysine residue in either of the two active sites of ALAS/ALAS has unique affects on the environment of the PLP cofactor of each site. These results indicate that each active site makes distinctive contributions to the UV/visible spectrum of the single chain dimeric ALAS/ALAS and that the linking of the two subunits of WT ALAS produces asymmetrical cofactor-binding sites in the single chain dimeric ALAS/ALAS.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Protein</th>
<th>LB/Amp/ALA</th>
<th>LB/Amp</th>
<th>LB/Amp</th>
<th>Biotin</th>
<th>minimal</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGF23</td>
<td>ALAS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pTDT1</td>
<td>AONS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pGF27</td>
<td>ALAS$_{K313A}$</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pTDT8</td>
<td>ALAS$_{F341A}$</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pAC1</td>
<td>ALAS/ALAS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pCA1</td>
<td>ALAS$_{K313A}$/ALAS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pMG1</td>
<td>ALAS/ALAS$_{K313A}$</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pTDT5</td>
<td>ALAS/AONS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pTDT4</td>
<td>AONS/ALAS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pTDT12</td>
<td>ALAS$_{K313A}$/AONS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pTDT17</td>
<td>AONS/ALAS$_{K313A}$</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>pTDT14</td>
<td>ALAS$_{F341A}$/AONS</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>pTDT15</td>
<td>AONS/ALAS$_{F341A}$</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
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<td>pTDT7</td>
<td>ALAS/AONS$_{K236A}$</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>
FIGURE 3.2
Absorption spectra of ALAS, ALAS/ALAS, ALAS$^{313A}$/ALAS and ALAS/ALAS$^{313A}$. The inset includes the region from 300–500 nm. Protein concentrations were adjusted to 30 μM for ALAS/ALAS, ALAS$^{313A}$/ALAS and ALAS/ALAS$^{313A}$ or 20 μM for ALAS in 20 mM Hepes, pH 7.5. ALAS/ALAS$^{313A}$ (—), ALAS$^{313A}$/ALAS (····), ALAS/ALAS(--), and ALAS (—·—).
Kinetic characterization of the $\text{ALAS}^{\text{K313A}}/\text{ALAS}$ and $\text{ALAS}/\text{ALAS}^{\text{K313A}}$ variants and determination of the dissociation constants for the binding of ALA.

The steady-state kinetics of the $\text{ALAS}^{\text{K313A}}/\text{ALAS}$ and the $\text{ALAS}/\text{ALAS}^{\text{K313A}}$ reactions were examined, and the results are presented in Table 3.3. Previously, it was reported that linking the two ALAS subunits into the single chain dimeric ALAS/ALAS resulted in over 5-fold and 28-fold increases in the $k_{\text{cat}}$ and $k_{\text{cat}}/K^\text{S\text{CoA}}_m$ values, respectively (Zhang, Cheltsov et al., 2005). In addition, mutagenesis of K313, the active site residue involved in the Schiff base linkage with the PLP cofactor (Ferreira, Neame et al., 1993) and in catalysis (Hunter and Ferreira, 1999), to alanine rendered the “natural” ALAS homodimer into a variant of the enzyme (K313A) with no measurable ALAS activity (Ferreira, Vajapey et al., 1995; Hunter and Ferreira, 1999). To determine the individual contribution of the two active sites to the overall steady-state activity of ALAS/ALAS, the steady-state kinetic parameters of the $\text{ALAS}^{\text{K313A}}/\text{ALAS}$ and $\text{ALAS}/\text{ALAS}^{\text{K313A}}$ variants, in which the K313A mutation was independently introduced in each of the two active sites, were determined (Table 3.3). While the K313A mutation in $\text{ALAS}^{\text{K313A}}/\text{ALAS}$ decreased the $k_{\text{cat}}$ 2.5-fold, the same mutation in $\text{ALAS}/\text{ALAS}^{\text{K313A}}$ resulted in a 12.6-fold decrease of the $k_{\text{cat}}$ value. Similarly, the catalytic efficiencies of $\text{ALAS}^{\text{K313A}}/\text{ALAS}$ for glycine and succinyl-CoA were decreased approximately 2.9- and 1.8-fold, respectively, whereas those of $\text{ALAS}/\text{ALAS}^{\text{K313A}}$ were reduced approximately 9- and 51-fold for glycine and succinyl-CoA, respectively. If both active sites were to contribute equally to the steady-state activity of ALAS/ALAS, then a 50% reduction of the turnover number would be expected for the single chain dimer in which the enzymatic activity of one of the two active sites was impaired. This seems to be the case for
ALAS\textsuperscript{K313A}/ALAS but not for the ALAS/ALAS\textsuperscript{K313A} variant. These findings suggest that linking the N-terminus of one subunit to the C-terminus of the other subunit in ALAS/ALAS may have created strain that hindered the steady-state enzymatic activity of one of the active sites, and, consequently, produced a single chain dimer with unequal participation of the two active sites.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (min(^{-1}))</th>
<th>$K_m^{Gly}$ (mM)</th>
<th>$k_{cat} / K_m^{Gly}$ (min(^{-1})mM(^{-1}))</th>
<th>$K_m^{SCoA}$ (μM)</th>
<th>$k_{cat} / K_m^{SCoA}$ (min(^{-1})μM(^{-1}))</th>
<th>$K_d^{ALA}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAS</td>
<td>10 ± 1(^a)</td>
<td>23 ± 1(^a)</td>
<td>0.43 ± 0.06(^a)</td>
<td>2.3 ± 0.1(^a)</td>
<td>4.3 ± 0.2(^a)</td>
<td>25 ± 3(^a)</td>
</tr>
<tr>
<td>ALAS/ALAS</td>
<td>55.4 ± 16.7 ± 0.2(^b)</td>
<td>3.32 ± 0.04(^b)</td>
<td>0.45 ± 0.03(^b)</td>
<td>123 ± 8(^b)</td>
<td>1.46 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>ALAS/ALAS\textsuperscript{K313A}</td>
<td>4.4 ± 0.2</td>
<td>11.8 ± 2.8</td>
<td>0.37 ± 0.09</td>
<td>1.8 ± 0.3</td>
<td>2.4 ± 0.4</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td>ALAS\textsuperscript{K313A}/ALAS</td>
<td>21.6 ± 0.7 14.8 ± 11.7</td>
<td>1.46 ± 0.17</td>
<td>0.32 ± 0.05</td>
<td>67.5 ± 10.8</td>
<td>2.3 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Data from ref (Gong, Hunter et al., 1998). \(^b\)Data from ref (Zhang, Cheltsov et al., 2005).
Pre-steady-state burst experiments for ALAS$^{K313A}$/ALAS and ALAS/ALAS$^{K313A}$.

Similarly to the ALAS-catalyzed reaction, the rate-limiting step for the single chain dimeric ALAS occurs after catalysis and is proposed to be associated with a protein conformational change associated with ALA release (Zhang, Cheltsov et al., 2005). To determine if the rate-limiting step of the reactions catalyzed by the K313A single chain dimeric variants occurs after the chemical step and to compare the amount of ALA produced in the first turnover of the ALAS$^{K313A}$/ALAS and ALAS/ALAS$^{K313A}$ reactions with that generated in the ALAS/ALAS first turnover, pre-steady-state burst experiments using chemical quenched-flow were performed (Figure 3.3). The reaction of ALAS$^{K313A}$/ALAS with saturating glycine and succinyl-CoA occurred with a burst in ALA production at a rate of $30.6 \pm 4.2$ s$^{-1}$, whereas the first turnover of the ALAS/ALAS$^{K313A}$ reaction was at a rate of $45.1 \pm 6.1$ s$^{-1}$. These burst rates are similar to the burst rate previously reported for the ALAS/ALAS reaction, $48.6 \pm 6.1$ s$^{-1}$ (Zhang, Cheltsov et al., 2005). Another important piece of information provided by the burst experiments shown in Figure 3.3 relates to the burst amplitudes, representing the concentration of ALAS active sites for the reactions. The burst amplitudes were 0.25/active site and 0.21/active site for ALAS$^{K313A}$/ALAS and ALAS/ALAS$^{K313A}$, respectively, nearly 50% less than the burst amplitude previously determined for ALAS/ALAS, 0.49/active site (Zhang, Cheltsov et al., 2005). These results are consistent with ALAS$^{K313A}$/ALAS and ALAS/ALAS$^{K313A}$ possessing half-sites of the ALAS/ALAS reactivity. Further, given that the number of the active sites of either ALAS$^{K313A}$/ALAS or ALAS/ALAS$^{K313A}$ is roughly 50% of that of ALAS/ALAS, the results also indicate
that the different contributions of each of the two active sites of ALAS/ALAS to its steady-state activity emanate from a step occurring after chemistry.
FIGURE 3.3
Kinetics of a pre-steady-state burst of ALA product in the ALAS/ALAS\textsuperscript{K313A} and ALAS\textsuperscript{K313A}/ALAS reactions. ALAS/ALAS\textsuperscript{K313A} (square) or ALAS\textsuperscript{K313A}/ALAS (triangle) (15 \textmu M) preincubated with glycine (200 mM) was quickly reacted with succinyl-CoA (150 \textmu M) at 20°C. The concentrations shown in parentheses are final concentrations after mixing. The reactions were quenched with 0.14 M perchloric acid at various aging times, and the ALA concentration was determined. The inset illustrates the first 0.3 seconds of the reaction time course. The curves represent the best fits to equation 1 with a burst amplitude of 6.27 ± 0.36 \textmu M and a burst rate of 45.1 ± 6.1 s\textsuperscript{-1} for ALAS/ALAS\textsuperscript{K313A} and a burst amplitude of 7.50 ± 0.31 \textmu M and a burst rate of 30.6 ± 4.2 s\textsuperscript{-1} for ALAS\textsuperscript{K313A}/ALAS.
Biological screening for ALAS and AONS function.

The determination of the crystallographic structures of ALAS (Astner, Schulze et al., 2005) and AONS (Alexeev, Alexeeva et al., 1998) confirmed the prediction drawn from the high degree of sequence similarity between ALAS and AONS (37%) that these two α-oxoamine synthases have similar 3D fold and active site architecture. To extend our studies on the plasticity of the PLP-binding and active site of ALAS to other members of the α-oxoamine synthase family, chimeras of ALAS and AONS and chimeras of singly-mutated ALAS variants and AONS were constructed (Table 3.1 and Figure 3.1). The two major objectives were 1) to determine whether the chimeras retain ALAS and AONS activities and 2) to examine the active site arrangement in the ALAS/AONS chimera. Both ALAS/AONS and AONS/ALAS exhibited ALAS and AONS activities as assessed by the positive genetic complementation of \textit{hemA}^− HU227 and \textit{bioF}^− R872 cells, which are ALA/heme (Sasarman, Surdeanu et al., 1968; Li, Brathwaite et al., 1989) and biotin auxotrophes (Del Campillo-Campbell, Kayajanian et al., 1967), respectively (Table 3.2). From these \textit{in vivo} activity assays, ALAS/AONS and AONS/ALAS appear to be bifunctional enzymes (\textit{i.e.} ALAS and AONS activities).

To start addressing the question related to the active site arrangement of ALAS/AONS chimera, directed-mutagenesis of critical residues in ALAS and AONS had to be established. K236 of \textit{E. coli} AONS, the conserved active site lysine involved in PLP binding and catalysis (Webster, Campopiano et al., 1998), corresponds to K313 of murine ALAS (Ferreira, Vajapey et al., 1995; Hunter and Ferreira, 1999). Thus, when the K236A mutation was introduced in the AONS homodimer, \textit{bioF}^− R872 cells did not grow in a medium without biotin (data not shown), similar to the absence of growth of \textit{hemA}^−.
HU227 cells overproducing ALAS$^{K313A}$ when plated onto a medium lacking ALA (Table 3.2). The crystal structure of the *R. capsulatus* ALAS homodimer revealed that amino acids of the two polypeptide chains contribute to the same active site, which is located at the dimer interface (Astner, Schulze et al., 2005). For example, F276 (*R. capsulatus* ALAS numbering or F341 in the equivalent murine erythroid ALAS numbering), a phenylalanine crucial for interaction with the pantetheine moiety of CoA, and the lysine involved in the Schiff base linkage participate in the architecture of the same active site but reside in different polypeptide chains. Of significance to this study, F341 is critical to ALAS function, as the F341A mutation abolished the production of ALA necessary to sustain growth of HU227 cells on a selective medium (Table 3.2). Thus, while K313 and F341 are critical for function of the murine erythroid ALAS homodimer, of the corresponding K236 and Y264 (Alexeev, Alexeeva et al., 1998), only K236 is essential for function in the *E. coli* AONS homodimer.

The three possible arrangements for the active sites of ALAS/AONS chimera are 1) formation of two active sites with the contribution of amino acids from one ALAS and one AONS polypeptide chains to each active site (Figure 3.4B) in an analogous arrangement to that of single chain dimeric ALAS/ALAS (Figure 3.4A); 2) formation of four active sites with the contribution of amino acids from one ALAS and one AONS polypeptide chains to each active site (Figure 3.4C); 3) formation of four active sites with two of the active sites being formed with only ALAS amino acids and the other two of the active sites with only AONS amino acids (Figure 3.4D), such that they represent WT ALAS- and AONS-like active sites. While the first active site arrangement would result from one single, chimeric polypeptide chain (Figure 3.4B), the latter two active site
arrangements would arise from two chimeric polypeptide chains (Figures 3.4C and 3.4D). The determined molecular mass of ~182 KDa for the chimeric ALAS/AONS (see below and Figure 3.5) ruled against a single chain, dimeric ALAS/AONS and the active site arrangement depicted in Figure 3.4B. To distinguish between the other two possibilities for the active site arrangement of the ALAS/AONS chimera (Figures 3.4C and 3.4D), an experimental approach involving the use of specific amino acid mutations targeted to abolish either ALAS or AONS function and biological selection systems (E. coli HU227 and E. coli R872) was followed. If the active site arrangement in Figure 3.4C were correct the K313A mutation would eliminate ALAS function in two of the four active sites, and the AONS residues in one or two of the remaining sites would have to complement the ALAS residues, yielding a chimeric protein with ALAS and AONS activities. In contrast, with the active site arrangement presented in Figure 4D, the ALAS and AONS activities arise from WT ALAS- and AONS-like active sites, and thus a deleterious mutation of a critical active site residue in ALAS or AONS would produce a chimeric enzyme with impaired ALAS or AONS activity. HU227 cells transformed with either pTDT12 or pTDT17 [i.e., expression plasmids for the ALAS/AONS chimera harboring the K313A mutation in ALAS (i.e., Table 3.1)] did not support the growth of these cells in an ALA-depleted medium (Table 3.2). However, R872 cells harboring either of these two plasmids could grow in a medium without biotin. A similar situation was observed with the chimeras ALAS$^{F341A}$/AONS and AONS/ALAS$^{F341A}$, in which the phenylalanine at position 341 of murine erythroid ALAS was substituted with an alanine (Table 3.2). When the ALAS sequence was maintained intact and a mutation of the Schiff base linkage-lysine residue was introduced into AONS (i.e., K236A), the
generated chimeric proteins, AONS$^{236\text{A}}$/ALAS or ALAS/AONS$^{236\text{A}}$, could rescue the growth of HU227 cells in a medium without ALA but not of R872 cells in a non-biotin supplemented medium (Table 3.2). Taken together, these findings are consistent with the active site arrangement for ALAS/AONS depicted in Figure 3.4D, in which the active sites responsible for ALAS activity are built with ALAS residues, while those with AONS activity are made of AONS residues.

Oligomeric state of the ALAS/AONS chimera.

Although ALAS/AONS and AONS/ALAS could be overproduced as active chimeric enzymes in E. coli DH5α cells (data not shown), only ALAS/AONS could be purified. Poor solubility and low stability were among the factors preventing the purification of AONS/ALAS.

The molecular mass of each subunit of the WT ALAS and WT AONS homodimers is ~56 and ~40 kD, respectively. Thus a single chimeric subunit, derived from the fusion of an ALAS and AONS polypeptide, is expected to be ~96 kD. This is in agreement with the molecular mass estimated by SDS-PAGE (Figure 3.5, inset). The molecular mass of the native ALAS/AONS chimera was determined to be ~182 kD (Figure 3.5), consistent of a “homodimer” of ~96 kD subunits. Further, coupled enzyme assays confirmed that the 182-kD protein exhibits both ALAS and AONS activities, indicating that the ALAS/AONS chimera is bifunctional as a homodimer of two ~96 kD chimeric polypeptide subunits.
FIGURE 3.4
Schematic representation illustrating the active site active site arrangement for ALAS/ALAS and possible active site arrangements for ALAS/AONS. Red bars represent ALAS polypeptide chains, with the essential active site residues K313 and F341 from one single polypeptide chain contributing to separate active sites. Green bars represent AONS polypeptide chains, with the essential active site residues K236 and Y264 from one single polypeptide chain contributing to separate active sites. In (A) and (B), there are two active sites per single chain dimer, whereas in (C) and (D), there are four active sites per chimeric ALAS/AONS dimer. (A) Active site arrangement of the ALAS/ALAS “single chain dimer”. (B) - (D) Possible active site arrangements for ALAS/AONS. (B) ALAS/AONS single chain dimer. (C) ALAS/AONS chimeric dimer with “hybrid” active sites comprised of both ALAS and AONS residues. (D) ALAS/AONS chimeric dimer with two of the active sites containing only ALAS residues and the other two active sites containing only AONS residues.
Spectroscopic characterization of AONS and ALAS/AONS.

At pH 7.5, absorbance maxima at ~330 and ~420 nm were also observed in the ALAS/AONS chimera (Figure 6A). Because the UV-visible spectra of both ALAS and AONS exhibited maxima at 330 nm and 420 nm, their fluorescence spectra were examined for distinctive features among ALAS, AONS, and ALAS/AONS. With excitation at 420 nm, the PLP cofactor of either ALAS or AONS exhibited a fluorescence emission maximum at 510 nm for ALAS and AONS (Figure 6B), albeit the magnitude of the 510 nm fluorescence emission maximum was more than three times greater for the AONS cofactor than that of ALAS. Similar to AONS and ALAS, the PLP cofactor of the ALAS/AONS chimera exhibited a fluorescence emission maximum at 510 nm upon excitation at 420 nm, and the magnitude of this fluorescence emission maximum fell between the values observed for the PLP cofactor of ALAS and AONS (Figure 6B). Upon excitation at 330 nm, the fluorescence emission spectra of ALAS and AONS exhibited maxima at 385 nm and 430 nm, respectively, while the fluorescence emission spectrum of the ALAS/AONS chimera displayed a broad emission band between 385 nm and 430 nm (Figure 6C).
FIGURE 3.5
Determination of the molecular mass of the ALAS/AONS chimera by gel filtration chromatography. ALAS/AONS (5 mg) was applied to a Pharmacia Sephadex 200 filtration column and eluted with 20 mM potassium phosphate buffer containing 10% glycerol at 4 °C and pH 7.5 (flow rate 1.0 mL/min). The molecular mass calibration curve for the Superdex 200 column using cytochrome c (12.4 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (45 kDa), bovine serum albumin (66.0 kDa), alcohol dehydrogenase (150.0 kDa), and β-amylase (200.0 kDa) as protein standards (indicated by circles). The ALAS/AONS chimera is indicated by a triangle. (Inset) 12.5% SDS-PAGE of the purified ALAS, AONS and ALAS/AONS chimera, which as were detected using Coomassie Brilliant Blue staining. Approximately 5 μg of each protein sample was loaded per lane.
FIGURE 3.6
Absorption and fluorescence spectra of ALAS, AONS and ALAS/AONS. (A) UV-visible absorption spectra. The inset includes the region from 300–510 nm. Fluorescence emission spectra upon excitation at (B) 420 nm and (C) 330 nm and fluorescence excitation spectra upon emission at 510 nm (inset B) and 385 nm (inset C). For absorption spectra, protein concentrations were adjusted to 15 μM (AONS and ALAS) or 7.5 μM (ALAS/AONS) and for fluorescence spectra, protein concentrations were adjusted to 4 μM (AONS and ALAS) or 2 μM (ALAS/AONS) in 20 mM Hepes, pH 7.5 containing 10% glycerol. For (A) – (C), AONS (→), ALAS (—), and ALAS/AONS (– –).
Steady-state and transient kinetics of ALAS/AONS.

To examine the ALAS and AONS activities of the ALAS/AONS chimera, the steady-state kinetic parameters of the chimeric protein associated with both activities were determined using substrates for ALAS and AONS (Tables 3.4 and 3.5). Regarding the ALAS activity, the $k_{cat}$ decreased almost 40%, the catalytic efficiency for glycine increased ~1.4-fold, and the catalytic efficiency for succinyl-CoA remained virtually the same relative to ALAS (Table 3.4). With respect to the AONS activity, while the value for $k_{cat}$ decreased approximately 50%, the catalytic efficiencies towards alanine and pimeloyl-CoA of the ALAS/AONS chimera were similar to those of AONS (Table 3.5).

Using chemical quenched-flow, pre-steady-state experiments of the ALAS reaction were performed to examine the extent of ALA production by ALAS/AONS and ascertain the reactivity of the ALAS active sites in the ALAS/AONS chimera (Figure 3.7). The time course associated with ALA formation was biphasic and is described by a burst phase with a rate of $13.2 \pm 2.6 \text{ s}^{-1}$ and a steady-state rate of $0.015 \text{ s}^{-1}$. While the values for the burst and the steady-state rates were approximately 70% and 50% of those previously determined for the ALAS-catalyzed reaction (Zhang, Cheltsov et al., 2005), the burst amplitude of 0.10/active site was similar to that formerly observed with ALAS (Zhang, Cheltsov et al., 2005). Significantly, the ~50% decrease in the value of the steady-state rate agrees with that inferred from the experiments performed under steady-state conditions (above). Thus, the reactivity of the ALAS sites in ALAS/AONS is similar to that of ALAS, and the diminished steady-state ALAS activity of the ALAS/AONS chimera in relation to that of ALAS must arise from a step occurring after
the reaction chemistry, presumably a strained protein conformational change associated with product release.
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<th>$K_m^{\text{Gly}}$ (mM)</th>
<th>$k_{cat} / K_m^{\text{Gly}}$ (min$^{-1}$mM$^{-1}$)</th>
<th>$K_m^{\text{SCoA}}$ (μM)</th>
<th>$k_{cat} / K_m^{\text{SCoA}}$ (min$^{-1}$μM$^{-1}$)</th>
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<td>ALAS$^a$</td>
<td>10 ± 1</td>
<td>23 ± 1</td>
<td>0.43 ± 0.02</td>
<td>2.3 ± 0.1</td>
<td>4.3 ± 0.2</td>
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<tr>
<td>ALAS/AONS</td>
<td>6.2 ± 0.8</td>
<td>5.5 ± 0.8</td>
<td>1.1 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>4.1 ± 0.7</td>
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$^a$Data from ref (Gong, Hunter et al., 1998)

<table>
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<th></th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m^{\text{Alanine}}$ (mM)</th>
<th>$k_{cat} / K_m^{\text{Alanine}}$ (min$^{-1}$mM$^{-1}$)</th>
<th>$K_m^{\text{PCoA}}$ (μM)</th>
<th>$k_{cat} / K_m^{\text{PCoA}}$ (min$^{-1}$μM$^{-1}$)</th>
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<tr>
<td>AONS$^a$</td>
<td>3.6 ± 0.6</td>
<td>0.5 ± 0.4</td>
<td>7.2 ± 1.3</td>
<td>25 ± 2</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>ALAS/AONS</td>
<td>1.7 ± 0.3</td>
<td>0.25 ± 0.05</td>
<td>6.8 ± 1.8</td>
<td>10 ± 1</td>
<td>0.16 ± 0.03</td>
</tr>
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</table>

$^a$Data from ref (Webster, Alexeev et al., 2000)
Figure 3.7
Kinetics of a pre-steady-state burst of ALA product in the ALAS/AONS reaction. ALAS/AONS (50 μM) preincubated with glycine (140 mM) was quickly reacted with succinyl-CoA (150 μM) at 20°C. The concentrations shown in parentheses are final concentrations after mixing. The reactions were quenched with perchloric acid (0.14 M) at various aging times, and the ALA concentration was determined. The inset illustrates the reaction time course, while the first 0.7 seconds of the reaction are shown in a larger plot. The first 0.7 s of the time course is expanded while in the inset the time course is extended to 3.0 s. The curve represents the best fit to equation 1 with a burst amplitude of $4.7 \pm 0.4$ μM and a burst rate of $13.2 \pm 2.6$ s$^{-1}$. 
Discussion

ALAS and other fold-type I PLP-dependent enzymes function as homodimers with two active sites per dimer; each active site is created at the interface between the two monomeric subunits (Christen and Mehta, 2001; Eliot and Kirsch, 2004). The crystal structure of the *R. capsulatus* ALAS holoenzyme revealed that the enzyme symmetrically binds two PLP molecules, one at each active site (Astner, Schulze et al., 2005). Previously, we demonstrated that linking the two subunits of ALAS into a single polypeptide chain yielded a more-active enzyme that functioned as a single-chain dimer (Zhang, Cheltsov et al., 2005). Although linking the N-terminus of one subunit and the C-terminus of the adjacent subunit without the introduction of a polypeptide linker did not affect the global conformation, changes in the environment of the PLP cofactor altered the predominant tautomeric form of the internal aldimine, which contributed to the greater activity of the single-chain dimer (Zhang, Cheltsov et al., 2005). However, it was not clear if these changes affected the enzymatic activity of each active site to a similar extent. To determine whether the two active sites in ALAS/ALAS contribute equally to enzymatic activity, we characterized variants in which one of the two active sites had no measurable enzymatic activity due to a mutation of the conserved K313 residue that binds to the cofactor.

Spectral characterization of and ALAS/ALAS\(^{K313A}\) and ALAS\(^{K313A}/\)ALAS revealed asymmetric cofactor environments in the two active sites, which was reflected in the disproportionate kinetic behavior of the two sites. In contrast to ALAS/ALAS and ALAS\(^{K313A}/\)ALAS, in which the steady-state rates increased 5-fold and 2-fold, respectively, relative to the values for WT ALAS, the steady-state rate decreased
approximately 50% for ALAS/ALAS\textsuperscript{K313A}. The concentration of catalytically active sites in ALAS/ALAS\textsuperscript{K313A} and ALAS\textsuperscript{K313A}/ALAS was half that of the ALAS/ALAS but, unlike the variations observed in the steady-state rates, the chemical rates were similar in all three forms. As with ALAS/ALAS and WT ALAS (Zhang and Ferreira, 2002; Zhang, Cheltsov et al., 2005), the pre-steady-state burst results for the two K313A variants were consistent with the rate-limiting step occurring after the reaction chemistry.

In ALAS, the rate-limiting step has been ascribed to the opening of an active site loop that allows ALA release; the rate of this assigned loop opening closely corresponds to the steady-state rate (Zhang and Ferreira, 2002; Hunter, Zhang et al., 2007). Thus, the variations observed in the steady-state kinetic parameters are likely to be due to alterations in the energy barrier for this conformational change required for ALA release. The ALAS crystal structure indicates that the N-terminus of one subunit and the C-terminus of the adjacent subunit are located near the surface on opposite faces of the holoenzyme (Astner, Schulze et al., 2005). The strain resulting from linking the remote N- and C-termini of two ALAS subunits in ALAS/ALAS appears to increase the barrier for product release at one site while decreasing the barrier at the other; that is, the steady-state enzymatic activity is enhanced at one active site and hindered at the other. Consequently, the active sites contribute asymmetrically to enzyme function.

Generally, the active sites in fold type I PLP-dependent enzymes are equivalent and independent (Eliot and Kirsch, 2004), although some examples of kinetic asymmetry have been documented. In glutamate-1-semialdehyde aminomutase (GSAM), allosteric interactions between active sites lead to inactivation of the site in one subunit by the activation of the site in the other subunit (Stetefeld, Jenny et al., 2006). In aspartate
aminotransferase (AAT), dissimilar lattice contact in the crystalline enzyme contributes to kinetic asymmetry in the active sites, although the active sites display kinetic equivalence in solution (Kirsten, Gehring et al., 1983). In addition, heterodimeric variants created for a number of complementation studies have contained asymmetric active sites (Onuffer and Kirsch, 1994; Tan and Ferreira, 1996; Tan, Harrison et al., 1998; Tarun and Theologis, 1998), but the creation of ALAS/ALAS is the first example of engineered active site asymmetry without the introduction of active site mutations in fold-type I PLP-dependent enzymes.

Despite the unequal steady-state rates in the functioning active sites of ALAS/ALAS\textsuperscript{K313A} and ALAS\textsuperscript{K313A}/ALAS, we would expect the total activity of the two variants to be similar to ALAS/ALAS, in which both sites contribute to enzyme function. Clearly, this was not the case. The total steady-state activities for the two K313A variants was only about 50% of the activity observed in the non-mutated single-chain dimer, suggesting that the enzymatic activity of at least one active site is impaired by the elimination of the Schiff base linkage between K313 and the PLP cofactor at the adjacent site. Conversely, studies in the unlinked ALAS\textsuperscript{K313A} and ALAS\textsuperscript{R149A} heterodimer, containing one nonactive site with both K313A and R149A mutations and one WT catalytically active site, show that the variant heterodimer retained approximately 50% of the activity observed in the ALAS homodimer (Tan and Ferreira, 1996); that is, the WT active site was not significantly affected by mutations at the adjacent site. Thus, linking the termini of the two ALAS subunits likely altered the intermolecular dynamics such that movement at one site can be transmitted to the adjacent site.
Because the single-chain ALAS dimer showed structural plasticity and had increased activity, we wondered whether the structural plasticity would extend to single-chain chimeras constructed from two members of the α-oxoamine synthase family, ALAS and AONS. We also hypothesized that it might be possible to generate an enzyme with novel activity by creating hybrid ALAS/AONS active sites. In vivo assays indicated that the ALAS/AONS and AONS/ALAS chimeras possessed both ALAS and AONS activities. Thus, the chimeric protein had sufficient structural plasticity to achieve the conformations necessary to produce both enzymatic activities. The linking of two ALAS subunits did not significantly affect the dimeric interface or the folding of the core in the individual subunits, and the dissociation of the dimeric interface of ALAS and the ALAS/ALAS single-chain dimer exhibited similar free energies and resulted in stable intermediates that retained a substantial amount of their secondary and tertiary structure (Cheltsov, Barber et al., 2001; Cheltsov, Guida et al., 2003). Similarly, we would not expect the linking of ALAS and AONS to strongly affect the folding core or subunit interface of the two domains, and it is not surprising that the two domains retained their overall structural character given that the entire ALAS and AONS polypeptides were used in the creation of the chimeras.

Despite our initial hypothesis that the chimeric protein would create chimeric active sites with potentially novel enzymatic activities, both ALAS/AONS and AONS/ALAS appeared to function as chimeric homodimers with functionally independent ALAS and AONS modules. In these modules, two active sites exhibiting ALAS activity were built exclusively with ALAS residues, and two active sites with AONS activity were built with AONS residues (Figure 3.4D). Nonetheless, the
dimerization of two chimeric polypeptides into a bifunctional homodimer with functionally independent ALAS and AONS modules suggests that the structural plasticity observed in ALAS can be extended to other members of the α-oxoamine synthase family. It is possible that creating a chimera from individual domains of each protein (for example, the N-terminal domain of ALAS and the catalytic and C-terminal domains of AONS) would produce a functional hybrid, but this remains to be tested.

Although we succeeded in expressing both the ALAS/AONS and AONS/ALAS chimeras as active enzymes, we were only able to purify the ALAS/AONS chimera. The molecular mass of the native ALAS/AONS chimera was consistent with that of a homodimer containing two chimeric polypeptide subunits. The fluorescence spectra exhibited by the ALAS/AONS chimera were distinct from those of ALAS and AONS and were consistent with an enzyme exhibiting a mixture of ALAS and AONS spectroscopic characteristics. The ALAS and AONS steady-state kinetic activities were diminished by roughly one-half in the chimera, and the catalytic efficiencies were not impaired. The pre-steady-state kinetic analysis for the ALAS reaction demonstrated that the reactivity of the ALAS sites in ALAS/AONS was similar to that of ALAS, with the rate-limiting step occurring after catalysis.

The linking of two proteins or functional domains in natural and de novo fusion proteins generally involves a peptide linker (Gokhale and Khosla, 2000; Arai, Ueda et al., 2001; Wriggers, Chakravarty et al., 2005). A primary goal of linker engineering is to effectively separate the two functional domains to avoid intermolecular strain and prevent unwanted interactions between the two modules (Carlsson, Ljung et al., 1996; Seo, Koo et al., 2000; Arai, Ueda et al., 2001; Arai, Wriggers et al., 2004; Wriggers, Chakravarty et
al., 2005). Because our objective was to facilitate interaction between the subunits of ALAS/ALAS and the AONS and ALAS chimeras, only two amino acids (Glu-Leu), which were introduced with the construction of a restriction site between the cDNAs, link the two subunits. Like both ALAS single-chain dimeric variants, the linking of the ALAS and AONS subunits appeared to change the energy barrier associated with the structural rearrangement that occurs upon ALA formation to allow product release. It is likely that the use of the short dipeptide to link the ALAS C-terminus with the N-terminus of either ALAS or AONS introduced intermolecular strain which altered conformational flexibility.

Remarkably, the strain introduced with the short linker did not significantly impair the catalytic efficiencies in the any of the fusion proteins. In fact, the catalytic efficiency for succinyl-CoA increased roughly 15- and 30-fold in ALAS$^{K313A}$/ALAS and ALAS/ALAS, respectively (Table 3.3). The short linker also appears to have facilitated interdomain communication in the single-chain dimeric variants, allowing changes at one site to be propagated to the adjacent site. Generally, efforts are made to avoid the introduction of strain when engineering hybrid proteins; however, with the increased use of high-throughput protein engineering, the introduction of intermolecular strain through linker domains may be a reasonable approach to creating new proteins with enhanced or novel functions.

In summary, we have shown that the two active sites in the ALAS/ALAS single-chain dimer make asymmetrical steady-state contributions to the activity of the enzyme. This and the reduced steady-state activity of the single-chain chimeras of ALAS and AONS relative to the parent enzymes are likely to be caused by differences in
conformational changes at product release, which in turn are due to the strain introduced by joining the two subunits without a linker region. Although the chimeric ALAS/AONS and AONS/ALAS proteins did not form hybrid active sites, they were able to form dimers with separate regions of ALAS and AONS activity. Thus, the extensive structural plasticity seen in ALAS extends to another member of the α-oxoamine family, AONS.
References


Chapter Four

Histidine-282 in 5-Aminolevulinate Synthase Affects Substrate Binding and Catalysis

Abstract

5-Aminolevulinate synthase (ALAS), the first enzyme of the heme biosynthetic pathway in mammalian cells, is member of the α-oxoamine synthase family of pyridoxal 5’-phosphate (PLP)-dependent enzymes. In all structures of the enzymes of the α-oxoamine synthase family, a conserved histidine hydrogen bonds with the phenolic oxygen of the PLP cofactor and may be significant for substrate-binding, PLP-positioning, and maintaining the pKa of the imine nitrogen. In ALAS, replacing the equivalent histidine, H282, with alanine reduces the catalytic efficiency for glycine 450-fold. The slow phase rate for glycine binding is decreased 60%, while the overall Kd increased 4.5 fold. The distribution of the absorbing 420 and 330 nm species was altered with an increased A420/A330 ratio from 0.45 to 1.05. This shift in species distribution was mirrored in the cofactor fluorescence and 300 to 500 nm circular dichroic spectra and likely reflects variation in the tautomer distribution of the holoenzyme. The 300 to 500 nm circular dichroic spectra of ALAS and H282A diverged in the presence of either glycine or aminolevulinate indicating that the reorientation of the PLP cofactor upon external aldimine formation is impeded in H282A. Alterations were also observed in the $K_{d}^{Gly}$ value and spectroscopic and kinetic properties, while the $K_{d}^{PLP}$ increased 9-fold.
Altogether, the results imply that H282 coordinates the movement of the pyridine ring with the reorganization of the active-site hydrogen bond network and acts as a hydrogen bond donor to the phenolic oxygen to maintain the protonated Schiff base and enhance the electron sink function of the PLP cofactor.

**Introduction**

Heme is an essential tetrapyrrole in nearly all living cells, and all tetrapyrroles are biosynthesized from the same precursor, 5-aminolevulinic acid (ALA). In mammals, 5-aminolevulinate synthase (ALAS, EC 2.3.1.37) catalyzes the condensation of glycine and succinyl-CoA to form ALA, CoA, and carbon dioxide, in the first and regulatory step of heme biosynthesis. Mammals express genetically distinct erythroid and housekeeping ALAS isoforms, and mutations in the erythroid specific ALAS have been implicated in X-linked sideroblastic anemia, a disease characterized by inadequate formation of heme and the accumulation of iron in the erythroblast mitochondria (May and Bishop, 1998).

ALAS belongs to a catalytically versatile class of enzymes that require pyridoxal 5’-phosphate (PLP) as a cofactor (Ferreira and Gong, 1995). PLP-dependent enzymes that catalyze reactions involving amino acids share common mechanistic characteristics based on utilization of the electron withdrawing properties of the cofactor to labilize bonds to the substrate α-carbon (Christen and Mehta, 2001). Specifically, the PLP cofactor covalently binds to the ε-amino group of an active site lysine via a Schiff base linkage to form the “internal aldimine.” The incoming amino acid substrate replaces the lysine amino group to form an “external aldimine” via a gem-diamine intermediate, in a reaction often referred to as transaldimination. Subsequently, the cleavage of one of the
substrate α-carbon bonds leads to a resonance-stabilized quinonoid intermediate in which the coenzyme acts an electron sink, storing electrons from the cleaved bond through the conjugated system of the Schiff base and pyridinium ring. Ultimately, the electrons are dispensed back for the formation of new linkages to the Cα (Christen and Mehta, 2001).

PLP-dependent enzymes have been classified according to reaction specificity relative to the Cα (Mehta and Christen, 1994) and fold-types derived from three-dimensional structures (Alexander, Sandmeier et al., 1994; Eliot and Kirsch, 2004). ALAS is classified within the α-oxoamine synthase sub-family of the α- within class II of fold type I of PLP-dependent enzyme superfamilies (Schneider, Kack et al., 2000). In all known structures of fold-type I, the pyridine ring of the PLP cofactor superimposes very well (Kack, Sandmark et al., 1999). The pyridoxal moiety interacts with the enzyme in a common motif, which includes the previously mentioned Schiff base linkage with an active site lysine, a salt-bridge between the pyridinium ring nitrogen and an aspartate, and a hydrogen bond with the phenolic oxygen which occurs through a variety of amino acids (Kack, Sandmark et al., 1999).

In ALAS and other α-oxoamine synthase enzymes, the hydrogen bond of the phenolic oxygen involves a conserved histidine (Alexeev, Alexeeva et al., 1998; Schmidt, Sivaraman et al., 2001; Astner, Schulze et al., 2005), which corresponds to H282 in murine erythroid ALAS (4.1). No studies have examined the role of this residue in any α-oxoamine synthase family member, although based on structural data alone it has been suggested that it may function as an acid catalyst during transaldimination (Webster, Alexeev et al., 2000; Zhang, Cheltsov et al., 2005), play a key role in positioning the PLP
aromatic ring (Schmidt, Sivaraman et al., 2001), or influence the $pK_a$ of the imine nitrogen (Webster, Alexeev et al., 2000).

Studies in other $\alpha$-family enzymes indicate that the significance of interaction between the protein and the phenolic oxygen of PLP may vary according to the requirements of the enzyme. In aspartate aminotransferase and 1-aminocyclopropane-1-carboxylate synthase, the phenolic oxygen interacts with a tyrosine residue (Goldberg, Swanson et al., 1991; White, Vasquez et al., 1994). The deletion of the hydrogen bond through the replacement of the active site tyrosine with phenylalanine reveals a different function in the kinetic properties of each enzyme. In aspartate aminotransferase, the tyrosine stabilizes the reactive form of the internal aldimine at physiological pH and increases the $k_{cat}$ value (Goldberg, Swanson et al., 1991; White, Vasquez et al., 1994). Similar studies of 1-aminocyclopropane-1-carboxylate synthase reveal that the tyrosine decreases the $K_m$, but has no affect on $k_{cat}$ (White, Vasquez et al., 1994).

In murine erythroid ALAS, H282 is tethered between PLP and Y121 through hydrogen bonds between the imidazole $N_{\epsilon2}$ and $N_{\delta1}$, respectively (Figure 4.1). Previous studies have demonstrated that the H-bond between the Y121 hydroxyl group and H282 $N_{\delta1}$ is important for efficient cofactor and substrate binding (Tan, Barber et al., 1998), providing evidence for a probable role for H282 in these interactions. The ordered ALAS catalytic pathway is comprised of the following steps (Scheme 4.1): the association of glycine with the enzyme forming the Michaelis complex (I); the transaldimination reaction between glycine and the active site lysine (K313) to generate the external aldimine (II); the removal of the pro-$R$ proton to generate a transient quinonoid intermediate (III); the condensation of succinyl-CoA (IV); the removal of Co-A and the
formation α-amino-β-keto adipate (V); decarboxylation of the α-amino-β-keto adipate(VI); the protonation of the second quinonoid intermediate (VII) and finally the releases of ALA (VII) (Zhang and Ferreira, 2002), (Hunter and Ferreira, 1999). In order to characterize the role of the conserved histidine in murine erythroid ALAS function, a series of H282 variants were constructed. The results provide evidence that H282 impacts a variety of ALAS functions including substrate and PLP binding and catalysis.

**Materials and Methods**

**Materials**

The following reagents were purchased from Sigma-Aldrich Chemical Company: DEAE-Sephacel, Ultrogel AcA-44, β-mercaptoethanol, PLP, bovine serum albumin, succinyl-CoA, ALA-hydrochloride, α-ketoglutaric acid, α-ketoglutarate dehydrogenase, Bis-Tris, HEPES-free acid, AMPSO-free acid, MOPS, tricine, thiamin pyrophosphate, NAD⁺, and the bicinchoninic acid protein determination kit. Glycerol, glycine, disodium ethylenediamine tetraacetic acid dihydrate, ammonium sulfate, magnesium chloride hexahydrate, perchloric acid, and potassium hydroxide were acquired from Fisher Scientific. Sodium dodecyl sulfate polyacrylamide gel electrophoresis reagents were acquired from Bio-Rad. Phenylhydrazine was from by Eastman Kodak. PD-10 columns were from Amersham Biosciences. Chameleon mutagenesis kit was from Stratagene. Xho I and Xba I restriction enzymes were from New England Biolabs.

**Methods**

**Mutagenesis**.
The pGF23 plasmid encoded the full-length sequence for the murine, mature erythroid ALAS. Site-directed mutagenesis for the H282Y and H282F mouse ALAS mutant was performed on the single-stranded pGF23 using the chameleon mutagenesis kit from Stratagene. The mutagenic oligonucleotides for H282Y and H282F were GAT GAA GTC TAT GCT TAT GCT GTA GGA CTG TAT GGA and GAT GAA GTC TTT GCT TAT GCT GTA GGA CTG TAT GGA, respectively, with the introduced codon substitutions underlined. The H282A mutant was generated using the method previously described by Gong (1998). Briefly, two rounds of PCR were performed to obtain DNA fragments with the desired mutation flanked by unique restriction sites. The mutagenic primers used to generate the H282A mutation were 5'-GTA GAT GAA GTC GCT GTA GGA CTG or 5'-GAG TCC TAC AGC AGC GAC TTC ATC TAC with the introduced codon substitution underlined. The two fragments containing the mutation were used as megaprimer and amplified by a third round of PCR. The product was then digested with Xba I and Xho I and subcloned into pGF23 vector. Clones obtained after mutagenesis procedures were confirmed by sequencing.

Protein purification, SDS-PAGE, protein determination and steady-state analysis

Recombinant murine erythroid ALAS and the H282A variant were purified from DH5α Escherichia coli bacterial cells containing the overexpressed protein as previously described (Hunter and Ferreira 1995). Sufficient expression of H282Y and H282F variants could not be obtained. Purity was determined by SDS-PAGE (Laemmli, 1970) and protein concentration determined by the bicinchoninic acid method using BSA as the standard (Smith, Krohn et al., 1985). All protein concentrations are reported on the basis of a subunit molecular weight of 56,000 kD. Enzymatic activity was determined by a
continuous spectrometric assay at 30°C (Hunter and Ferreira, 1995). To evaluate the pH
dependence of the kinetic parameters, assays were performed in 20 mM MOPS for pH
6.7, HEPES for pH 7-8 or AMPsO for pH 8.2-9.5. The pH dependences of log $k_{\text{cat}}$ and
log $k_{\text{cat}}/K_m$ were fit to equation 1, while the pH dependence of log $1/K_m$ was fit to
equation 2.

Equation 1 \[ \log Y = \log \frac{Y_{\text{max}}}{1 + 10^{pH - pK_{a,1}} + 10^{pK_{a,2} - pH}} \]

Equation 2 \[ \log Y = \log \frac{Y_{\text{max}}}{1 + 10^{pK_{a} - pH}} \]

Spectroscopic measurements.

Prior to spectroscopic measurement, enzyme was dialyzed in 20 mM HEPES, pH
7.5 with 10% glycerol to remove free PLP. Absorption spectra were acquired at ambient
temperature using a Shimadzu UV 2100 dual beam spectrophotometer, with a reference
containing all components except the purified enzyme. Circular dichroism (CD) spectra
were obtained using an AVIV CD spectrometer calibrated for both wavelength maxima
and signal intensity with an aqueous solution of D-10 camphorsulfonic acid (Chen,
1977). Protein concentrations were 10-11 μM and 100 μM for the near and far CD
spectra, respectively, in 20 mM Bis-Tris, pH 7.5 containing 10% glycerol. Spectra were
recorded in triplicate and averaged, using a 0.1 cm path length cuvette with a total
volume of 300 μl. Fluorescence spectra were collected on a Shimadzu RE-5301 PC
spectrofluorophotometer using protein concentrations of 2-4 μM. The pH was adjusted
with 20 mM MOPS (pH range 6.7-7.0), 20 mM HEPES (pH range 7- 8.2), or 20 mM

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AMPSO, (pH range 8.3-9.5). 10% glycerol was also included in the buffers. CD and fluorescence blank spectra were collected from samples containing all components except protein immediately prior to the measurement of samples. The blank spectra were subtracted from spectra of sample containing enzyme. The pH dependence of the 510 nm-fluorescence emission upon 420 nm-excitation was fit to equation 3.

\[
Y = \frac{Y_{\text{max}} - Y_{\text{min}}}{1 + 10^{(\alpha - \rho K)}} + Y_{\text{min}}
\]

**Stopped-flow spectroscopy.**

Rapid scanning stopped-flow measurements were conducted using a model RSM-100 stopped-flow spectrophotometer (OLIS Inc. This instrument has a dead-time of approximately 2-ms and an observation chamber path length of 4 mm. Scan spectra covering a wavelength range of 300-510 nm were collected at a rate of 1000 scans/s and then averaged to 62 scans/s to reduce data files to a manageable level. The temperature of the syringes and the stopped-flow cell compartment was maintained at 30°C by an external water bath. The concentration of glycine was always at least 10-fold greater than the enzyme concentration to ensure pseudo-first order kinetic were observed.

For each experimental condition, three replicate experiments were performed. The \(\Delta\text{Absorbance} 420\ \text{nm}\) were globally fit using the simulation software Dynafit to the binding models described in scheme 4.2 and 4.3 (Kuzmic, 1996).
Determination of dissociation constants of glycine and ALA.

Dissociation constants were determined spectroscopically by monitoring spectral changes upon the binding of glycine and ALA (Gong, Hunter et al., 1998). The $K_d$ values for glycine from pH 6.7-9.5 were determined at 30 °C for ALAS and the H282A variant by monitoring the increase in cofactor absorbance at 420 nm upon glycine binding. The pH was adjusted with 20 mM MOPS (pH range 6.7-7.0), 20 mM HEPES (pH range 7-8.2), or 20 mM AMPSO, (pH range 8.3-9.5). 10% glycerol was also included in the buffers. Glycine was prepared as 2 M stocks adjusted to the same pH as the corresponding buffers. $K_d$ is defined by equation 4 where [Gly] and [Enz] are the concentrations of free glycine and free enzyme, respectively, and [Gly-Enz] represents the concentration of glycine-bound ALAS.

$$K_d = \frac{[\text{Gly}][\text{Enz}]}{[\text{Gly} - \text{Enz}]}$$

The changes in absorbance at 420 nm were plotted as a function of glycine concentration and the data were fit to equation 5 to determine $K_d$, where $\Delta$Abs is the absorbance increase at 420, $Abs_{\text{max}}$ is the maximum increase in absorbance, and [Gly] is
the total glycine concentration. The pH dependence of $K_d$ for ALAS was fit to *equation 3* and for H282A *equation 6*.

\[
\Delta \text{Abs} = \frac{\text{Abs}_{\text{max}} [\text{Gly}]}{K_d + [\text{Gly}]} \\
\
\text{Equation 5}
\]

\[
Y = \frac{Y_{\text{max}}}{1 + 10^{pH - pK_{s1}} + 10^{pK_{s2} - pH}}
\]

\[
\text{Equation 6}
\]

The ALA $K_d$ for the H282A variant was determined by monitoring the decrease in absorbance at 420 nm at 30°C in 20 mM HEPES, pH 7.5 and 10% glycerol. Enzyme (25-30 μM) solution was titrated with small aliquots of concentrated ALA solution, and the change in absorbance measured. Data were analyzed by non-linear regression fitting to *equation 7*, where $A$ is the observed absorbance, and $A_i$ and $A_f$ are the fitted values of the initial and final absorbance, respectively. $[L]$ is the ligand concentration, and $[E]$ is the enzyme concentration. Determinations were made in duplicate and the reported values represent the mean and standard error of measurement.

\[
\text{Equation 7} \quad A = A_i + \left[ \frac{(A_f - A_i) \times K_d + [L] + [E] - \sqrt{(K_d + [L] + [E])^2 - 4[L][E]}}{2[E]} \right]
\]
Preparation of apoenzyme and determination of the PLP dissociation constant.

To obtain H282A apoenzyme, 1 mg/ml enzyme in 20 mM HEPES pH 7.5, containing 20% glycerol was treated with 150 mM phenylhydrazine for 1.5 hours at 4°C, following which phenylhydrazine was removed by running the solution through a PD-10 column. The phenylhydrazine treatment was then repeated to ensure all PLP was removed.

The PLP $K_d$ for the H282A variant was determined at 25°C by monitoring the PLP-dependent increase in 510 nm fluorescence emission upon excitation at 420 nm, in a buffer composed of 20 mM HEPES, pH 7.5 and 10% glycerol. To determine $K_d$, data were analyzed by non-linear regression fitting to equation 7, where $A$ is the observed fluorescence, and $A_i$ and $A_f$ are the fitted values of the initial and final fluorescence, respectively.

**pH titration of quinonoid intermediate formation for H282A variant.**

The pH dependence of quinonoid intermediate formation was investigated with ALA saturated enzymes as described previously (Gong, Hunter et al., 1998). Equation 8 was used to fit the quinonoid intermediate titration curves where $Y$ is the observed absorbance at 510 nm, $Y_{max}$ and $Y_{min}$ are the theoretical maximal and minimal absorbance values at 510 nm, and $pK_a$ is the equivalence point for quinonoid intermediate formation.

$$Y = \frac{Y_{max} - Y_{min}}{1 + 10^{(pK_a - pH)}} + Y_{min}$$
Results

Spectroscopic properties of the H282A variant.

At pH 7.4, three absorbance maxima at approximately 278, 330 and 420 nm are observed in both ALAS and the H282A variant (Figure 4.2A). The absorbance at 278 nm is primarily due to the protein, while the 330 nm and 420 nm maxima are common in PLP-dependent enzymes and are typically attributed to deprotonated and protonated aldimine species, respectively (Metzler and Metzler, 1987). A similar assignment for ALAS is ambiguous because the spectrum is unchanged in the pH range 6.5-9.5. The mutation had no discernable effect on the protein absorption band centered at 278 nm, but the cofactor absorption peaks were significantly altered. The ratio of the 420 nm to 330 nm absorbance was increased from 0.45 in the wild-type enzyme to 1.05 in the variant.

The changes in the absorption spectra were reflected in the fluorescence spectra (Figure 4.2B and C). Upon excitation at 330 nm ALAS exhibits only one maximum at 385 nm, while in H282A the 385 nm fluorescence emission maximum is shifted to 410 nm with a 6-fold decrease and a second maximum is observed at 510 nm. With excitation at 420 nm, the cofactor exhibits fluorescence emission maximum at 510 nm for both enzymes; however the magnitude of the 510 nm emission was ~7 times greater in the H282A. The pH titration of the species emitting at 510 nm upon excitation at 420 nm demonstrated that this species diminished as a result of loss of a single proton for both enzymes (Figure 4.3). A fit of the data to equation 3 yield a pK_a of 8.05 ± 0.043 and 9.02 ± 0.07 for ALAS and H282A, respectively.

1 G.C. Ferreira and G.A. Hunter, unpublished results
Kinetic characterization of the H282A variant.

The steady-state kinetic parameters of the H282A variant were determined and the results are summarized in Table 4.1. The mutation resulted in a $k_{\text{cat}}$ of 1.4% of the wild-type ALAS value. The $K_m$ for glycine was increased 5-fold relative to ALAS, while the $K_m$ for succinyl-CoA was not significantly affected. The overall catalytic efficiency for glycine and succinyl-CoA decreased 450-fold and 87-fold respectively as compared to ALAS values.

If H282 acts as a hydrogen bond donor to the phenolic oxygen of the cofactor, then the H282A mutation may lower the $pK_a$ for the imine nitrogen. To investigate this possibility the pH dependence of the steady-state kinetic parameters was studied, with the results summarized in Table 4.2 and Figure 4.4. The log $k_{\text{cat}}$ vs. pH profile for H282A decreased on both the acidic and basic sides, and the best fit of the data to equation 1 generated a $pK_a$ of 7.2 ± 0.1 for a residue in the enzyme-substrate complex that must be protonated for optimal catalysis. A second $pK_a$ of 8.6 ± 0.1 for a residue that is deprotonated during catalysis was also observed for H282A, which shifted from the previously reported $pK_a$ of 9.1 ± 0.03 in ALAS (Zhang, Cheltsov et al., 2005). The possibility of an acidic limb $pK_a$, below 7.0, in the wild-type enzyme could not be investigated due to instability at pH values below 6.5-7.0 (Zhang, Cheltsov et al., 2005), but the available data do suggest that the H282A mutation results in a substantial increase to the $pK_a$ of an important enzyme-substrate complex ionization. This ionization might be assigned directly to H282, or it could be assigned to the imine nitrogen that presumably shares a proton with the phenolic oxygen atom.
The log $k_{cat}/K_m$ pH profile for the mutant was similar to that of the wild-type enzyme, decreasing on both the acidic and basic limbs. Nonlinear regression of the data using equation 1 yielded a p$K_a$ for the acidic and basic limb of 8.00 ± 0.14 and 8.50 ± 0.14 for H282A, which reflects a shift in the acidic limb from the at 8.60 ± 0.11 p$K_a$ value previously reported in ALAS (Zhang, Cheltsov et al., 2005). The pH variation of the log $1/K_m^{Gly}$ decreased with increasing pH for both enzymes. The data were fit to equation 2 to generate a p$K_a$ of 8.36 ± 0.1 for ALAS and 7.76 ± 0.16 for the H282A variant. The log $k_{cat}$ and log $k_{cat}/K_m^{Gly}$ profiles limiting slopes of approximately 1 or -1 indicate the ionization of a single group for acidic and basic limbs. Given that glycine is not a sticky substrate and does not ionize over the pH range studied, the p$K_a$ observed for $1/K_m^{Gly}$ and the acidic limb of the log $k_{cat}/K_m^{Gly}$ likely represents group(s) in the free enzyme.
Table 4.1: Summary of steady-state kinetic parameters and dissociation constants

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m^{Gly}$ (mM)</th>
<th>$k_{cat}/K_m^{Gly}$</th>
<th>$K_m^{Gly}$ (mM)</th>
<th>$K_m^{SCoA}$ (μM)</th>
<th>$K_m^{ALA}$ (μM)</th>
<th>$K_d$ (μM)</th>
<th>$k_{cat}/K_m^{SCoA}$</th>
<th>$K_d^{PLP}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAS$^a$</td>
<td>10 (±1)</td>
<td>23 (±1)</td>
<td>0.4 (±0.06)</td>
<td>22 (±2)</td>
<td>2.3 (±0.1)</td>
<td>4.35 (±0.62)</td>
<td>25 (±3)</td>
<td>0.05 (±0.002)</td>
<td>40 (±4)</td>
</tr>
<tr>
<td>H282A</td>
<td>0.137 (±0.003)</td>
<td>144 (±7.7)</td>
<td>9.5 x10$^{-4}$ (±0.06 x10$^{-4}$)</td>
<td>49 (±5)</td>
<td>2.75 (±0.07)</td>
<td>0.05 (±0.002)</td>
<td>40 (±4)</td>
<td>14 (±4)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Data from (Gong, 1998)

Table 4.2: Summary of pK values obtained from the pH dependence of kinetic parameters

<table>
<thead>
<tr>
<th>Protein</th>
<th>pK$^a$</th>
<th>pK$^b$</th>
<th>pK$^c$</th>
<th>pK$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAS</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H282A</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Log</th>
<th>$k_{cat}$</th>
<th>9.1±0.03</th>
<th>7.2±0.09</th>
<th>8.6±0.09</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log</td>
<td>$1/K_m^{Gly}$</td>
<td>8.4±0.10</td>
<td>7.8±0.16</td>
<td></td>
</tr>
<tr>
<td>Log</td>
<td>$k_{cat}/K_m^{Gly}$</td>
<td>8.6±0.11$^a$</td>
<td>8.75±0.13$^b$</td>
<td></td>
</tr>
<tr>
<td>Log</td>
<td>$K_d^{Gly}$</td>
<td>nd (&lt;7)</td>
<td>7.4±0.2</td>
<td>8.1±0.2</td>
</tr>
</tbody>
</table>

$^a$Data from (12)  
$^b$Data from (21)

nd, not determined
Reaction of glycine with H282A variant.

The reaction of 60 μM H282A variant with glycine resulted in an increased absorbance at 420 nm (Figure 4.5a). The data best fit to the two step model described by scheme 4.2 (Figure 4.5b-c). A fit of the data yielded values for $k_1$ of $0.001654 \pm 3.8 \times 10^{-5}$ s$^{-1}$, $k_2$ of $0.14 \pm 0.0064$ s$^{-1}$, $k_2$ of $0.022 \pm 0.0025$ s$^{-1}$, and $k_{-2}$ of $0.0455 \pm 0.0016$.

Dissociation constants for the binding of glycine and ALA.

To elucidate a potential role of H282 in substrate binding, the enzymes were titrated with glycine and ALA to determine the dissociation constants for formations of the external aldimine with the substrate and product. At pH 7.5 the $K_d$ for ALA and glycine increase 8.5-fold and 5-fold, respectively, relative to ALAS (Table 4.1). To establish if the ionization of groups reflected in the $k_{cat}$ profiles are involved in substrate binding or catalysis, the pH dependence of $K_d$ for glycine was determined. The loss of the PLP-O3-H282 interaction also had a marked effect on the pH profile for the $K_d^{Gly}$ values. For ALAS, the $K_d^{Gly}$ decreases with increasing pH and, when fitted to equation 1, yielded a $pK_a$ value at the boundary of the pH range tested, therefore a $pK_a \leq 7$ was assumed. In contrast, the $K_d^{Gly}$ for the H282A variant fit to a bell curve with $pK_a$ values at $7.4 \pm 0.2$ and $8.1 \pm 0.2$ (Figure 4.6). The data indicate that the H282 mutation results in a substantial modification to the $pK_a$ of an enzyme-glycine complex ionization.

pH titration of quinonoid intermediate formation for H282A variant.

When ALAS is saturated with ALA, the external aldimine is converted to a quinonoid intermediate in a pH-dependent manner; the extent of this reaction can be monitored by following the absorbance of the quinonoid intermediate at 510 nm. Formation of the ALA-bound quinonoid intermediate in ALAS has been reported to
occur with an apparent $pK_a$ of $8.1 \pm 0.1$ (Gong, Hunter et al., 1998), and involves participation of the active site K313, which acts as a general base catalyst for the reaction by abstracting a proton from the ALA-aldimine to form the quinonoid intermediate (Hunter and Ferreira, 1999). The ALA-bound quinonoid intermediate was observed to increase with pH for H282A as was observed previously in ALAS (Hunter and Ferreira, 1999), although the amplitude of the absorption of the quinonoid intermediate was markedly diminished by the mutation at all pH values tested (Figure 4.7A). pH titration of the H282A quinonoid intermediate absorbance demonstrated that the intermediate was formed as a result of loss of a single proton with an equivalence point at $8.8 \pm 0.1$ (4.7B). The higher $pK_a$ value in the variant indicates that one function of H282 is to lower the apparent $pK_a$ for quinonoid intermediate formation such that the PLP cofactor functions more effectively as an electron sink at physiological pH. The observation that disruption of a hydrogen bond to the phenolic oxygen of the cofactor has a significant effect on quinonoid intermediate formation indicates that the equivalence point of $8.1 \pm 0.1$ observed with ALAS is a complex function of the electronic interaction of the active site lysine with the ALA-PLP aldimine and its active site environment, and not simply reflective of an ionization constant for the active site lysine.

**CD spectroscopy.** The disruption of the H-bond between the phenolic oxygen and the enzyme could potentially alter the time-averaged orientation of the PLP cofactor in the active site. The circular dichroism in the UV-visible region reflects the PLP microenvironment by monitoring the asymmetry of the bound cofactor. Formation of an external aldimine results in the reorientation of the PLP cofactor which can be followed with CD spectroscopy (Moore, Dominici et al., 1995). Spectra of the holo- and ligand-
bound enzymes were collected (Figure 4.8). Spectra of the free enzyme exhibited positive dichroic bands at ~330 and 420 nm with an increase in the 420 nm band with an associated decrease in the ~330 nm band observed in the variant. The addition of glycine to ALAS or H282A resulted in comparable decrease in the ~330 nm dichroic band, while the ~420 nm band decreased 75% in the variant and disappeared in the ALAS spectra. The addition of a saturating concentration of ALA to ALAS or H282A had strikingly different effects on the relative chiral environment of the external aldimine in the two enzymes. Specifically, the ~330 nm dichroic band was decreased and the ~420 nm band disappeared in the ALAS spectra, while the addition of ALA to H282A resulted in a moderate increase in the 330 nm and little change to the ~420 nm band. The CD spectra for ALAS and H282A between the 200-300 nm were similar, indicating that no significant changes occurred in the overall conformation as a result of the mutation (data not shown).

_Dissociation constants for the binding of PLP._

To address the role of H282 in cofactor binding, the effect of the H282 to alanine mutation on $K_d$ of PLP was studied. The titration of the H282A apoenzyme with PLP leads to the reconstitution of the holoenzyme, which can be monitored by following changes in the intensity of fluorescence emission at 510 nm upon excitation at 420 nm (Figure 4.6). Theoretical saturation curves were generated from which the dissociation constant of PLP from H282A was determined. When compared to the wild-type enzyme, the $K_d$ for PLP was increased ~9 fold by the H282A mutation, as reported in Table 4.1.
**Discussion**

The crystal structure of *Rhodobacter capsulatus* ALAS reveals the existence of a hydrogen bond between H282 and the phenolic oxygen atom of the PLP cofactor (Astner, Schulze et al., 2005). A clustal sequence alignment demonstrated that this histidine residue was perfectly conserved in over 70 known ALAS sequences from bacteria to mammals (data not shown). The existence of one, and often two, hydrogen bonds between the enzyme and the PLP phenolic oxygen is common in fold type I PLP-dependent enzymes, and is likely multifunctional. The ALAS crystal structures suggest possible roles for H282 in binding and orientation of the cofactor within the active site, as well as control of the electronic status of the cofactor during catalysis (Alexeev, Alexeeva et al., 1998). These possibilities led us to postulate that mutation of H282 should have multiple effects on substrate and cofactor binding, as well as catalysis. In this communication, we constructed ALAS variants harboring the H282A, H282Y, and H282F mutations, of which only the H282A variant was recoverable as a soluble enzyme. The effects of the H282A mutation on the spectroscopic and kinetic properties of the enzyme were characterized in order to better understand the functional roles of H282 in the ALAS-catalyzed formation of ALA.

The absorption spectra (Figure 4.2) indicate the mutation has a substantial effect on the electronics of the PLP cofactor. A decrease in the absorbance of the 330 nm peak is accompanied by an increase in the absorbance of the 420 nm peak. These changes are reflected in the cofactor fluorescence spectra. In some transaminases, including aspartate aminotransferase and tyrosine aminotransferase, the corresponding absorbance peaks titrate as a function of pH with the long wavelength peak favored at low pH and the
short wavelength peak favored at high pH (Goldberg, Swanson et al., 1991; Chow, McElroy et al., 2004) These are generally attributed to the ketoenamine and enolamine tautomers, respectively, which differ in the position of the proton shared between the phenolic oxygen and the Schiff base nitrogen atoms. The changes in the absorbance spectra for H282A suggest that the mutation significantly alters the equilibrium of cofactor tautomeric structures to favor the ketoenamine, but this assignment is ambiguous because, unlike aspartate and tyrosine aminotransferases, the absorbance spectrum of ALAS is largely pH-independent and the H282A mutation did not alter this property (data not shown).

In contrast to the absorption spectra, fluorescence spectra of ALAS upon excitation at 330 or 420 nm are pH-dependent (Zhang, Cheltsov et al., 2005). Upon excitation at 420 nm, the ALAS 510 nm fluorescence emission titrates with a single $pK_a$ of $8.05 \pm 0.043$, while in H282A a $pK_a$ of $9.02 \pm 0.07$ is observed under similar conditions (Figure 4.3). The 385 nm fluorescence emission signal resulting from excitation at 330 nm, which occurs with a $pK_a$ of $8.4 \pm 0.1$ in the wild-type enzyme, is greatly diminished in the mutant, and an equivalent titration could not be performed.

The two $pK_a$s observed in ALAS fluorescence spectra are presumably indicative of more complex chemistry than simple titration of the Schiff base nitrogen atom. This is not unprecedented, as in dialkylglycine decarboxylase, three $pK_a$s are observed during absorbance spectra titrations, with both ketoenamine and enolamine species present in each ionization state (Zhou and Toney, 1999). For both dialkylglycine decarboxylase and glutamate decarboxylase it has been proposed that the multiple ionizations observed reflect active site residues that regulate the distribution of ketoenamine and enolamine
tautomers through electrostatic effects (Chu and Metzler, 1994; Zhou and Toney, 1999).
In ALAS, the ionizations observed in the fluorescence spectra, but not the absorption spectra, are also likely to be attributable to active site residues and not the Schiff base nitrogen. Alterations observed in the H282A spectra may be due to changes in both tautomeric equilibria and the electrostatic interactions between the phenolic oxygen and other active site residues.

The steady-state kinetic parameters of the variant indicate loss of H282 interaction with the phenolic oxygen impairs both glycine binding and catalysis. The $K_m^{\text{Gly}}$ increased 5-fold and the $k_{\text{cat}}$ decreased by two orders of magnitude. Rapid-scanning stopped-flow analysis experiments were performed to further characterize the effect of the mutation. A pre-steady-state burst of the quinonoid intermediate for the reaction of H282A-glycine and succinyl-CoA was not observed, presumably due to diminished absorption of the quinonoid intermediate that is typically observed with the wild-type enzyme (data not shown).

The transimination reaction expected to occur during glycine binding involves nucleophilic attack of the protonated Schiff base internal aldimine by the deprotonated amine of glycine, to form a transient gem-diamine intermediate. If the hydrogen bond donated by H282 to the phenolic oxygen of the cofactor is important in maintaining a protonated Schiff base, then the loss of this hydrogen bond in H282A might be expected to slow the rate at which glycine binds to the enzyme. In the absence of succinyl-CoA, glycine binding to H282A is a two-step process (Figure 4.5). Previous studies demonstrated that glycine also binds with ALAS in two steps; however, the rates associated with the fast phase were not slow enough to be resolved (Hunter and Ferreira,
The slow phase rate for H282A decreased 85% relative to ALAS. The slower binding of glycine observed in H282A may be attributed to alterations in the electronic status of the Schiff base, but other interpretations are also possible. One interesting possibility is that H282 is directly or indirectly involved in proton transfers that convert the internal aldimine and glycine to the reactive ionic states necessary to formation of the glycine external aldimine (Scheme 4.1, I). In any case, these data, along with the data in Figure 4.6, indicate an important role for H282 in glycine binding.

The pH-dependence of log $k_{\text{cat}}$, log $k_{\text{cat}}/K_m^{\text{Gly}}$ and log $1/K_m^{\text{Gly}}$ were all diminished in H282A-catalyzed reaction, indicating that the mutation had severe catalytic consequences (Figure 4.4), but only the log $k_{\text{cat}}$ profile contained an ionization that was obviously changed by the mutation. The appearance of a new pK$_a$ of ~7.3 for H282A in the acidic limb of both the log $k_{\text{cat}}$ and log $K_m^{\text{Gly}}$, suggests that the mutation results in a substantial change to a pK$_a$ for the enzyme–glycine complex. In ALAS $k_{\text{cat}}$ is known to be determined by release of ALA, or a conformational change associated with ALA release (Hunter and Ferreira 1999). The appearance of an acid limb ionization in the log $k_{\text{cat}}$ vs. pH profile for H282A shifted the pH optimum from less than 6.5 to slightly over 8.0, and indicates a change in the nature of the rate-determining step for catalysis, at least at lower pH values. The further observation that a similar pK$_a$ is apparent in the log $K_m^{\text{Gly}}$ pH profile suggests that in H282A the rate-determining step at pH values less than 8.0 may be associated with binding of glycine. The ALAS spectroscopic pK$_a$ of 8.4 ± 0.1 observed upon excitation at 330 nm is mirrored in the log $1/K_m$ and the acidic limb of log $k_{\text{cat}}/K_m$ pH profile of ALAS (Zhang, Cheltsov et al., 2005). Although it was not possible
to titrate the equivalent species in the H282A spectra, the pH dependence of the log $1/K_m$ and acidic log $k_{cat}/K_m$ was shifted to ~7.9. This ionization controls the reactive free enzyme species, and the disappearance or significant reduction of the equivalent species in the H282A spectrum suggests H282 stabilizes the reactive form of the internal aldime.

In ALAS, the binding of ALA results in the appearance of an ALA-quinonoid intermediate with a 510 nm absorbance (Gong, Hunter et al., 1998). In H282A the addition of ALA results in a decrease in absorbance at 420 nm with an associated increase at 330 nm in addition to the appearance of a 510 nm absorbance, though the amplitude of the 510 nm absorption associated with the ALA-quinonoid species is markedly diminished. While $K_d$ for ALA is minimally affected, the $pK_a$ of the ALA-quinonoid intermediate is increased from 8.1 ± 0.1 in ALAS to 8.8 ± 0.1 in H282A. The data suggest that proton abstraction from ALA is impaired in the variant. One possible explanation is that the loss of the hydrogen bond between the phenolic oxygen and H282 is likely to cause a net flow of electrons into the conjugated $\pi$-bond system, thereby disrupting the electron sink capacity of the cofactor.

Additionally, it has been suggested that the process of ALA binding and quinonoid intermediate formation may involve some structural reorganization of the active site (Hunter and Ferreira, 1999). In both the glycine and succinyl-CoA soaked *R. capsulatus* ALAS crystals, a $15^\circ$ rotation of the pyridine ring around the C5-C5A bonds occurs such that the O3 and C4A atoms move away from the catalytic lysine (Astner, Schulze et al., 2005). Upon the binding of product in AONS, a similar rotation of the pyridine ring occurs along with subtle rearrangement of the active site hydrogen bond.
system (Webster, Alexeev et al., 2000). In ALAS, the movement of the O3 is tracked by the residue equivalent to H282 (Astner, Schulze et al., 2005), indicating H282 is probably involved in coordinating the movement of the pyridine ring with the reorganization of the hydrogen bond system occurring upon substrate binding. H282 is tethered between the phenolic O3 of PLP and Y121 by way of hydrogen bonds between the imidazole Nε2 and Nδ1, respectively. The loss of H282 hydrogen bonds with the cofactor and Y121 would likely affect the PLP movement and orientation within the active site that are presumably a crucial aspect of the catalytic process.

The possibility that the PLP microenvironment is affected in solution by the H282A mutation was examined using CD spectroscopy, performed in the absence and presence of ALA or glycine. The spectra for the ALAS and H282A holoenzymes are relatively similar and exhibit two positive dichroic bands around 330 and 420 nm (Figure 4.8) which mirror the shift from the 330 to 420 nm species observed in the absorbance spectra (Figure 4.2). In these holoenzyme spectra the cofactor is covalently anchored to the enzyme via the internal aldimine linkage with the active site lysine, and this attachment would be expected to maintain the orientation of the cofactor in the active site. However, upon formation of an external aldimine with glycine or ALA, the attachment of the cofactor to the active site lysine is lost, and the resulting CD spectra diverge in the two enzymes. Upon the addition of glycine, the 420 nm dichroic band disappears in ALAS, while the band continues to be observed in the H282A spectra (4.8). In ALAS, the binding of ALA results in the loss of the 330 nm band and a significantly diminished ~420 nm band. In contrast, the binding of ALA to H282A results in a spectrum remarkably similar to the holoenzyme. The divergence observed in
the ligand bound CD spectra of ALAS and H282A suggest that the reorientation of the PLP cofactor, observed with ALAS upon external aldime formation, is blocked or diminished by the H282A mutation. This would influence both the cofactor position and interaction with key catalytic residues and could help explain the multiple effects caused by the mutation in the variant.

In summary, H282 is involved in a hydrogen bond with the phenolic oxygen of the PLP cofactor. The deletion of this interaction in the H282A variant has multiple effects on the spectral, binding, and kinetic properties of the enzyme that support the conclusion that H282 plays multiple roles in the enzymology of ALAS. It may also be further concluded that the impaired function of the variant results from a combination of direct and indirect effects, including alterations in the protonation of the phenolic oxygen and changes to the stereoelectronic relationships between the cofactor and active site residues, through the disruption in the processional PLP positioning that normally occurs during catalysis.
Scheme 4.1
FIGURE 4.1
Spatial position of active site residues in the *R. capsulatus* ALAS holoenzyme crystal structure. This view highlights the interaction of the pyridinium ring of the cofactor with active site residues and the H282 imidazole N$_{\varepsilon2}$ and N$_{\delta1}$ hydrogen bonds between the cofactor phenolic oxygen and Y121. The image was constructed using Pymol (DeLano 2002) and PDB file 2BWN. Residue numbering is relative to murine erythroid ALAS.
FIGURE 4.2
Absorption and fluorescence spectra of ALAS and H282A variant. (A) UV-visible absorption spectra. The inset includes the region from 250–300 nm. Protein concentrations were adjusted to 13 μM in 20 mM Hepes, pH 7.5. (B) Fluorescence emission spectra of 5 μM ALAS and H282A in 20 mM Hepes, pH 7.5 containing 10% glycerol upon excitation at (B) 330 nm and (C) 420 nm. For (A) – (C), ALAS (---) and H282A (—).
FIGURE 4.3
The pH dependence of fluorescence emission. The fluorescence emission at 510 nm upon excitation at 420 nm for 2.0 μM ALAS (▲) and 3.5 μM H282A (●) at varying pH. Each of the line represents the nonlinear regression fit to equation 3.
FIGURE 4.4
pH dependence of (A) log $k_{\text{cat}}$, (B) log $k_{\text{cat}}/K_m^{\text{Gly}}$ and (C) log $1/K_m^{\text{Gly}}$ for ALAS (- -) and H282A (—). The lines represent the nonlinear regression fits to equation 1 or 2 as described in Materials and Methods. The profiles for the pH dependence of the steady-state kinetic parameters for ALAS (- -) are from (Zhang, Cheltsov et al., 2005).
FIGURE 4.5
Reaction of 60 μM H282A variant with glycine. (A) Spectra changes observed during the reaction of 300 mM glycine with H282A. Spectra were collected at 1, 5, 11, 23, 41 and 52 seconds and are shown sequentially with the lowest to the highest absorbance at 420 nm. The ΔA_{420} were globally fit to a two-step model using the simulation software Dynafit. (B) The ΔA_{420} data for the time course reaction of 300 mM glycine is represented by circles, with the line representing the fitted data. (C) The fit of the ΔA_{420} data for glycine binding at 100, 125, 150, 200, 300, 400, 500 and 600 mM.
FIGURE 4.6
pH dependence of the $K_d$ for glycine for ALAS (▲) and H282A (●). The data were fit to equation 5 (ALAS) or equation 6 (H282A) using non-linear regression analysis.
FIGURE 4.7
UV-visible absorption spectra of H282A in the presence of ALA and pH-dependence of ALA-quinonoid intermediate formation. (A) Absorption spectra of H282A (---) and ALAS (---) in the presence of 500 μM ALA. (Inset) Absorption spectra of H282A (---) and ALAS (---) in the presence of 300 μM ALA. Spectra were acquired at 30 °C and pH 7.5. (B) pH-dependence of quinonoid intermediate absorption upon addition of 20 mM ALA to either ALAS (---) or H282A (---). The lines represent theoretical curves based on the best fit of the data to equation 8.
FIGURE 4.8
Circular dichroism spectra of ALAS- and H282A-ligand complexes. Spectra of ALAS and H282A (A) Holoenzymes; (B) in the presence of 200 mM glycine; (C) in the presence of 300 μM ALA. Spectra were recorded in 20 mM Bis-Tris with 10% glycerol, pH 7.5, at an enzyme concentration of 100 μM.
Supporting Information

Histidine-282 in 5-Aminolevulinate Synthase Affects Substrate Binding and Catalysis

**FIGURE 4.9**
The $\Delta A_{420}$ were globally fit to a two-step model using the simulation software Dynafit. The $\Delta A_{420}$ data for the time course reaction of varying glycine concentrations represented by circles, with the lines representing the fitted data.
References


Chapter Five

Summary and Conclusions

Sequence and phylogenetic analysis of PLP-dependent $\alpha$-oxoamine synthases and their role in identifying residues regulating enzyme specificity

The CoA-dependent acyltransferases or $\alpha$-oxoamine synthases constitute a small but widespread reaction-specific subfamily within the $\alpha$-family of PLP-dependent enzymes; it is comprised of ALAS, AONS, SPT and KBL (Alexeev, Alexeeva et al., 1998; Schneider, Kack et al., 2000; Schmidt, Sivaraman et al., 2001; Astner, Schulze et al., 2005; Yard, Carter et al., 2007). Here we perform phylogenetic analysis of the $\alpha$-oxoamine synthase subfamily of PLP-dependent enzymes to understand the evolutionary progression of functional specialization and facilitate a better understanding of the mechanisms by which these enzymes enforce selectivity.

Sequences were selected to represent the phylogenetic and taxonomic distribution of each member of the $\alpha$-oxoamine synthase subfamily of PLP-dependent enzymes. Using the sequence alignment of the selected sequences, the evolutionary history was inferred and phylogenetic trees were constructed using three methods. The data are consistent with the appearance of AONS function early in the evolutionary time line of the $\alpha$-oxoamine synthases with the subsequent development of ALAS, KBL, SPT and SPT1/2 function. This is not surprising given that AONS was likely the only $\alpha$-oxoamine
synthase present in the universal ancestor cell and that AONS is one of only five PLP-dependent enzymes found in all biological kingdoms (Mehta and Christen, 1998).

Although the phylogenetic analysis was initially performed as a tool to help characterize the significance of amino acid changes in laboratory-evolved ALAS variants that were not realized, the alignments were utilized to identify residues in positions that may be significant for the regulation substrate specificity. Of the 37 residues identified, all but four were located at or adjacent to the active site. Interestingly, one third of the residues identified by our analysis were located at key active site positions in the ALAS crystal structure from *R. capsulatus* and/or were previously recognized to be mutated in ALAS-2 in patients with x-linked sideroblastic anemia.

**Functional Asymmetry for Active Sites of Single Chain Homo- and Chimeric Dimers of 5-Aminolevulinate Synthase and 8-Amino-7-Oxononanoate Synthase**

To determine whether the two active sites in ALAS/ALAS contribute equally to enzymatic activity, we characterized variants in which one of the two active sites had no measurable enzymatic activity due to a mutation of the conserved K313 residue that binds to the cofactor. Spectral characterization of ALAS/ALAS\(^{K313A}\) and ALAS\(^{K313A}/\)ALAS revealed asymmetric cofactor environments in the two active sites, which was also reflected in the disproportionate kinetic behavior of the two sites.

The pre-steady-state burst results for the two K313A-containing variants indicate that the chemical rates were similar to ALAS/ALAS and WT ALAS and, like ALAS/ALAS and WT ALAS, were consistent with the rate-limiting step occurring after the reaction chemistry (Zhang and Ferreira, 2002; Zhang, Cheltsov et al., 2005).
ALAS, the rate-limiting step has been ascribed to a conformation change that occurs prior to the release of the ALA product (Zhang and Ferreira, 2002; Hunter, Zhang et al., 2007). The strain resulting from linking the remote N- and C-termini of two ALAS subunits appears to increase the energy barrier for product release at one site while decreasing the barrier at the other; that is, the steady-state enzymatic activity is enhanced at one active site and hindered at the other. Consequently, the active sites contribute asymmetrically to enzyme function.

Because the single-chain ALAS dimer showed structural plasticity and had increased activity, we wondered whether the structural plasticity would extend to single-chain chimeras constructed from two members of the α-oxoamine synthase family, ALAS and AONS. Both ALAS/AONS and AONS/ALAS chimeras had sufficient structural plasticity to achieve the conformations necessary to produce both enzymatic activities.

Despite our initial hypothesis that the chimeric protein would create chimeric active sites with potentially novel enzymatic activities, both ALAS/AONS and AONS/ALAS appeared to function as chimeric homodimers with functionally independent ALAS and AONS modules. Nonetheless, the dimerization of two chimeric polypeptides into a bifunctional homodimer with functionally independent active sites suggests that the structural plasticity observed in ALAS can be extended to other members of the α-oxoamine synthase family.

The ALAS/AONS chimera was purified for further analysis. The fluorescence spectra exhibited by the ALAS/AONS chimera were consistent with an enzyme exhibiting a mixture of ALAS and AONS spectroscopic characteristics. The ALAS and
AONS steady-state kinetic activities were diminished by roughly one-half in the chimera, and the catalytic efficiencies were not impaired. The pre-steady-state kinetic analysis for the ALAS reaction demonstrated that the reactivity of the ALAS sites in ALAS/AONS was similar to that of ALAS, with the rate-limiting step occurring after catalysis.

Like ALAS/ALAS, the linking of the ALAS and AONS subunits appeared to change the energy barrier associated with the structural rearrangement that occurs upon ALA formation to allow product release. It is likely that the use of the short dipeptide to link the ALAS C-terminus with the N-terminus of either ALAS or AONS introduced intermolecular strain which altered conformational flexibility. Our studies involving the chimeras between ALAS and AONS demonstrate that the extensive structural plasticity seen in ALAS extends to another member of the α-oxoamine family, AONS.

**Histidine-282 in 5-Aminolevulinate Synthase Affects Substrate Binding and Catalysis**

A clustal sequence alignment demonstrated that a histidine residue was perfectly conserved in over 70 known ALAS sequences from bacteria to mammals and this histidine was also conserve among the α-oxoamine synthases. In murine ALAS-2, this conserved histidine corresponds to H282. The crystal structure of *Rhodobacter capsulatus* ALAS reveals the existence of a hydrogen bond between the equivalent histidine and the phenolic oxygen atom of the PLP cofactor (Astner, Schulze et al., 2005). A series of H282 murine ALAS-2 variants were constructed to characterize the role of this conserved residue, however H282A was the only variant recoverable as a soluble enzyme. Though this residue was predicted to have multiple roles, including functioning as an acid catalyst during transaldimination (Webster, Alexeev et al., 2000; Zhang,
Cheltsov et al., 2005), positioning the PLP aromatic ring (Schmidt, Sivaraman et al., 2001), and regulating the pKₐ of the imine nitrogen (Webster, Alexeev et al., 2000), we were surprised by the range of effects on the spectral, binding and kinetic properties that resulted from the replacement of H282 with alanine.

The absorption and fluorescent spectra indicated that the mutation had a substantial effect on the electronics of the PLP cofactor and suggest that the mutation significantly alters the equilibrium of cofactor tautomeric structures to favor the ketoenamine. The steady-state kinetic parameters of the variant revealed that the loss of the H282 interaction with the phenolic oxygen impairs both glycine binding and catalysis, reducing the catalytic efficiency for glycine 4505-fold. The slow phase rate for glycine binding in H282A decreased 60% relative to ALAS, while the overall kinetic Kₐ increased 4.5 fold. The rate-determining step is also altered in the H282A and is likely associated with glycine binding (at pH below 8.0) and not ALA release, as is observed in ALAS. The pH dependence of the log 1/Kₐ and acidic log kₗ/Kₐ provides evidence that H282 stabilizes the reactive form of the internal aldimine and is consistent with the change in tautomeric structures observed in the absorbance spectra. In H282A the ALA-quinonoid species is markedly diminished, while Kₐ for ALA is minimally affected.

In the R. capsulatus crystal structure, H282 hydrogen bonds with both the phenolic oxygen of the PLP cofactor and Y121. We suspected that the loss of the H282 hydrogen bonds with the cofactor and Y121 likely affect the PLP movement and orientation within the active site impacting multiple aspects of the catalytic process. Using CD spectroscopy, we determined that while the PLP microenvironment in the holoenzyme was similar in ALAS and H282A, the microenvironment diverged upon the
binding of the 5-aminolevulinate product and glycine substrate. This alteration in the cofactor microenvironment would impact both cofactor position and interactions with key catalytic residues. Therefore, we conclude that the multiple effects of the loss resulting from the loss of H282 results from a combination of direct and indirect effects, including alterations in the protonation of the phenolic oxygen and changes to the stereoelectronic relationships between the cofactor and active site residues, through the disruption in the processional PLP positioning that normally occurs during catalysis.
References


About the Author

Tracy D. Turbeville was born in 1970 in Lakeland, Florida. She graduated *cum laude* from the University of South Florida in 1997, earning a Bachelor’s degree Secondary Science Education with a specialization in Biology. As an undergraduate, Tracy was a recipient of the Woods Undergraduate Research Fellowship sponsored by the Institute for Biomolecular Science. Tracy entered the Interdisciplinary Ph.D. Program in Cellular and Molecular Biology, IP2CMB, and joined Dr. Gloria Ferreira’s laboratory in the Department of Molecular Medicine in the College of Medicine at the University of South Florida. While a graduate student, she received a Burroughs Wellcome Tuition Bursary to attend a Canadian Bioinformatics Workshop, earning a Certificate in Protein Informatics. Tracy served as the Association of Medical Sciences Graduate Students (AMSGS) Vice President and Secretary, as well as acting as the AMSGS representative to the Graduate Student and Professional Student Council.