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Molecular enzymology of 5-Aminolevulinate synthase, the gatekeeper of heme biosynthesis[☆]

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Abstract

Pyridoxal-5'-phosphate (PLP) is an obligatory cofactor for the homodimeric mitochondrial enzyme 5-aminolevulinate synthase (ALAS), which controls metabolic flux into the porphyrin biosynthetic pathway in animals, fungi, and the α -subclass of proteobacteria. Recent work has provided an explanation for how this enzyme can utilize PLP to catalyze the mechanistically unusual cleavage of not one but two substrate amino acid α -carbon bonds, without violating the theory of stereoelectronic control of PLP reaction-type specificity. Ironically, the complex chemistry is kinetically insignificant, and it is the movement of an active site loop that defines k_{cat} and ultimately, the rate of porphyrin biosynthesis. The kinetic behavior of the enzyme is consistent with an equilibrium ordered induced-fit mechanism wherein glycine must bind first and a portion of the intrinsic binding energy with succinyl-Coenzyme A is then utilized to perturb the enzyme conformational equilibrium towards a closed state wherein catalysis occurs. Return to the open conformation, coincident with ALA dissociation, is the slowest step of the reaction cycle. A diverse variety of loop mutations have been associated with hyperactivity, suggesting the enzyme has evolved to be purposefully slow, perhaps as a means to allow for rapid up-regulation of activity in response to an as yet undiscovered allosteric type effector. Recently it was discovered that human erythroid ALAS mutations can be associated with two very different diseases. Mutations that down-regulate activity can lead to X-linked sideroblastic anemia, which is characterized by abnormally high iron levels in mitochondria, while mutations that up-regulate activity are associated with X-linked dominant protoporphyria, which in contrast is phenotypically identified by abnormally high porphyrin levels. This article is part of a Special Issue entitled: Pyridoxal Phosphate Enzymology.

Keywords

Pyridoxal-5'-phosphate; Aminolevulinate synthase; Enzyme mechanism; Catalysis; Heme biosynthesis

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1. Introduction

ALAS1 (EC 2.3.1.37) catalyzes the PLP-dependent reaction of glycine with succinyl-Coenzyme A to produce Coenzyme A, carbon dioxide, and ALA, the latter of which is the common committed precursor of all biological tetrapyrroles, including hemes, chlorophylls, and cobalamins [1,2]. ALAS is found exclusively in non-plant eucaryotes and the α -subclass of proteobacteria. Plants and other bacteria do not code for an ALAS and in these organisms ALA is instead produced by the evolutionarily related PLP-dependent enzyme glutamate-1-semialdehyde 2,1-aminomutase, which catalyzes the single substrate *intramolecular* transamination of glutamate-1-semialdehyde to ALA [3,4].

ALAS activity couples heme and cytochrome production to aerobic respiration via common utilization of succinyl-Coenzyme A, as illustrated in Fig. 1. This reaction is both the key regulatory and rate-determining step of porphyrin biosynthesis [2]. Consequently, alterations in ALAS activity have marked effects on porphyrin production. In animals two chromosomally distinct and differentially regulated ALAS genes are present, one of which is devoted to hemoglobin biosynthesis in developing erythrocytes (ALAS-2), and the other of which is expressed in all tissues for production of cytochromes and other hemoproteins (ALAS-1) [2]. Excluding the mitochondrial import sequences, the two human ALAS gene products share 74% identity at the amino acid level.

1.1. An intriguing evolutionary significance

The differential production of ALA in plants and animals provides a very interesting area for speculation on the key evolutionary branch points between these kingdoms of organisms. Whereas animals utilize ALA to produce hemoglobin and other hemoproteins primarily for aerobic respiration, plants preferentially employ this metabolite in the biosynthesis of chlorophylls for photosynthesis. In both cases PLP-dependent ALA formation plays a central role in energy acquisition and utilization by the host organism [5,6]. ALAS is nuclear encoded and targeted to mitochondria, which are most closely related to α -proteobacteria, the only procaryotes known to code for ALAS [7]. This suggests that ALAS may have been an important factor in the natural selection of these particular bacteria as mitochondrial progenitors. In plants glutamate-1-semialdehyde 2,1-aminomutase is also nuclear encoded but is instead targeted to chloroplasts [5]. These organelles are evolutionarily derived from cyanobacteria, which obtain their energy via photosynthesis [7]. The structurally based finding that ALAS likely evolved from glutamate-1-semialdehyde 2,1-aminomutase would arguably identify a primary feature of the momentous divergence in eucaryotic metabolism away from utilizing metalloporphyrins to harvest photon energy, and instead focusing on stepwise reduction of molecular oxygen to water [8].

1.2. Notable structural features of ALAS

ALAS has been classified as a member of the α -family of PLP-dependent enzymes [9], which catalyze transformations of amino acids wherein the bonding rearrangements occur at the substrate α -carbon atom [10]. ALAS was initially categorized as part of the aminotransferase fold-type I subfamily branch of the α -family tree, based on sequence alignments [11], but recent analyses based on crystallographic data indicate that it is more appropriately considered to be part of the fold-type II subfamily branch [8]. Similar to the related L-amino acid transaminases, ALAS is a homodimer wherein the active sites reside at the subunit interface (Fig. 2) [12].

¹The abbreviations used are: ALAS, 5-aminolevulinate synthase; ALA, 5-aminolevulinate; PLP, pyridoxal-5'-phosphate.

Currently the only crystal structures of ALAS available are for the enzyme from *Rhodobacter capsulatus*, but with 49% sequence identity and 70% similarity to the catalytic core of human ALAS there is little doubt that these structures provide excellent models for the catalytic cores of all ALASs [13]. Not only has the holoenzyme structure been solved, but also the structures with either glycine or succinyl-CoA bound [13]. Alignment of these substrate-bound structures permits construction of an excellent model for the Michaelis complex, and the open active site loop in the holoenzyme structure allows visualization of just how this loop enforces an induced-fit type mechanism and must move away from the active site cleft in order to allow ALA dissociation [14]. The *R. capsulatus* ALAS crystal structures have proven to be invaluable aids to robust interpretation of kinetic and spectroscopic data obtained with the purified recombinant murine ALAS-2 enzyme.

The PLP cofactors delineate the area of the active site where the bonding rearrangements occur, and are very near the center of the three-dimensional structure. Although the adenine ring of succinyl-Coenzyme A binds near the enzyme surface, the pantothenate portion of the molecule extends down a cleft towards the cofactor, over 20 Å from the surface. This solvent excluded active site environment appears exquisitely evolved to ensure highly specific molecular recognition of succinyl-Coenzyme A, while the area where glycine binds is so spatially constrained that even L- or D-alanine will not substitute as alternative substrates. At the apex of the active site loop T4302 forms a strong 2.4–2.5 Å low barrier hydrogen bond with the carboxylate tail of succinyl-Coenzyme A upon adoption of the closed conformation (Fig. 5) [15]. T430 is in turn held rigidly in place by bracketing proline residues at positions 429 and 432, and despite being approximately 10 Å away from the center of the PLP cofactor this residue is clearly essential for effective catalysis, as even the conservative T430S mutation has been found to be associated with X-linked sideroblastic anemia [16,17].

2. ALAS reaction chemistry

Jencks [18] suggested that God created PLP especially for enzymologists who enjoy pushing electrons, and to the extent this might be true ALAS should provide ample opportunity for entertainment, as the chemical mechanism is not only complex, but also unusual and enlightening. Two-dimensional depictions of the reaction chemistry such as that in Fig. 3 give a good indication of the complexity, yet fail to convey the precise stereoelectronic control that is believed to be involved in substrate recognition and the conversion of substrates to products. In fact, ALAS would probably provide a good model system for examining the seemingly never ending question as to the extent to which an enzyme can “freeze out” torsional motions of the reactants and translate this so called “entropy trap” into rate enhancements [19–21].

The ALAS reaction chemistry is unusual for a PLP-dependent enzyme in that not one, but two substrate amino acid α -carbon bonds are cleaved during the reaction cycle, as if it were both a transaminase and a decarboxylase. This was for many years difficult to reconcile with the generally accepted theory that reaction-type specificity of PLP α -carbon substrate bond-cleavage is under strict stereoelectronic control [22–24]. The stereoelectronic control hypothesis (sometimes referred to as “Dunathan's hypothesis”) states that upon formation of an external aldimine the relative lability of the three substrate amino acid α -carbon bonds is crucially dependent upon their orientation relative to the planar and electron withdrawing cofactor π -bonding system. Cleavage only occurs rapidly when a bond is aligned perpendicular to the plane of the PLP-aldimine conjugated system. This three-dimensional stereoelectronic orientation most closely aligns the σ -orbitals of the bond to be cleaved with the π -orbitals of the cofactor, and since these orbitals must overlap and rehybridize for any

²Ferreira, G.C., Zhang, J., Lendrihas, T., Gong, J., and Hunter, G. A., unpublished observations.

reaction to occur, the favorable positioning accelerates the rate of cleavage over the out-of-alignment α -carbon bonds, by a factor that has been estimated to be approximately a million-fold [25,26]. Thus, an important function of PLP-dependent enzymes in general is to simply bind the external aldimine complex such that the target bond is oriented perpendicular to the plane of the cofactor, thereby optimally exposing this single bond to the electron sink functionality of the cofactor, while simultaneously providing a means of discriminating against side reactions. Cleavage of more than one bond during a reaction cycle, as in the ALAS or dialkylglycine decarboxylase reactions [27], therefore implies that there must be some intermediate torsion about the α -carbon bond between reaction steps, an alternate reaction pathway that does not utilize the electron sink property of the cofactor, or that the stereoelectronic control hypothesis is flawed or incomplete. In the case of dialkylglycine decarboxylase it is essentially the first of these possibilities, torsion about the α -carbon bond between decarboxylation and transamination half-reactions, that was found to be operative [24]. In the ALAS reaction, however, it is the second possibility, an alternate reaction pathway, which explains how the enzyme catalyzes multiple α -carbon bond rearrangements. Thus the two unusual PLP-dependent enzymes support the theory of stereoelectronic control, but each in a different way.

The overall reaction essentially involves the PLP-dependent replacement of the carboxyl group of glycine with a succinyl group donated from succinyl-CoA, but the mechanistic pathway is far more involved than a PLP chemist might envision, as seen in Fig. 3. The evolutionary relationship to α -amino acid transaminases is mechanistically manifested in utilization of the conserved active site lysine residue, that forms an internal aldimine with PLP in the absence of amino acid substrates, to catalyzes proton transfers following formation of external adimines with glycine or ALA [28,29]. The stereospecific cleavage of the pro-*R* bond of glycine and ALA carbon-5 by ALAS corresponds to recognition of these achiral stereocenters as it they were in fact the *L*-isomers. Upon formation of the external aldimine with glycine the binding of succinyl-Coenzyme A triggers accelerated removal of the pro-*R* proton of glycine by lysine-313, (structures II–III in Fig. 3) [29–31]. Kinetic and structural data converge upon the conclusion that succinyl-Coenzyme A binding promotes an induced fit type mechanism involving movement of the highly conserved active site loop over the active site such that the cofactor, substrates, and key enzyme residues are brought into optimal apposition for catalysis to occur [13,31–33]. Crystallographic data from succinyl-Coenzyme A bound *R. capsulatus* ALAS indicate that in three out of four monomers the PLP cofactor is released from the active site lysine and is present as the free aldehyde, further suggesting that binding of this substrate facilitates reaction of the cofactor with glycine, as previously demonstrated kinetically [13,31].

During stopped-flow experiments the reaction from structures II to V occur over approximately ten milliseconds at 30 °C, and the quinonoid intermediate (III) forms and decays too rapidly to be directly observed, and can only be detected spectroscopically when glycine is replaced by O-methyl glycine³ [34]. Reaction with succinyl-Coenzyme A yields an unstable tetrahedral intermediate (IV), which rapidly collapses to form an external aldimine with α -amino- β -keto adipate (V). Decarboxylation of this intermediate produces a remarkably stable and pH-titratable quinonoid intermediate (VII) [29,35], but due to the stereoelectronic constraints discussed above the decarboxylation step does not directly utilize the cofactor as an electron sink, and instead proceeds via an enol (VI) formed by β -keto acid decarboxylation. This step is acid catalyzed with an apparent pK_a of 7.7 ± 0.1 by H207, which resides almost directly above the cofactor, as depicted in Fig. 4 [32]. The enol is in turn in rapid equilibrium with an unusually stable quinonoid intermediate (VII), as well

³Lendrihas, Hunter, and Ferreira, unpublished observations.

as the product bound external aldimine formed by protonation of the enol by K313 (VIII) [29]. Ironically, the quinonoid intermediate that has been the key spectroscopic signal used to study the enzyme chemistry is not directly on the reaction pathway! ALA is then released in the slowest step of the reaction to complete the cycle. The remarkable precision of these steps is evidenced by kinetic isotope data with deuteroglycine as substrate, which indicated that the deuterium removed from glycine at the beginning of the reaction cycle is, at least in a significant proportion of turnovers, returned back to the ALA product, and is not exchanged with protons from water or the enzyme during the catalytic cycle [32].

3. ALAS kinetic mechanism

Although the ALAS reaction chemistry may be complex, it is not kinetically significant. Instead it is movement of the active site loop towards the open conformation (Fig. 5) that controls the rate at which ALA dissociates, and therefore the overall catalytic rate [14,31,32,35,36]. This is depicted in Scheme 1, which records the rates and equilibrium constants for the kinetic mechanism, along with the assigned reaction steps and the conformational status of the enzyme at each step. The binding of substrates and formation of the 1-amino-2-ketoadipate intermediate external aldimine are rapid, and in stopped-flow experiments the first positive signal observed is formation of a quinonoid intermediate coincident with the rate of product formation as determined by quench-flow [31,32,35]. This has been interpreted as indicating that the time taken to go from structures I to V in Fig. 3 is no more than approximately five milliseconds, and it is the subsequent steps that dominate the overall kinetics. Thus the kinetic mechanism of ALAS diverges substantially from the chemical mechanism [32].

The slow rate of the conformational change is coincident with k_{cat} [14,16] and presumably confers some important selective advantage, as a surprisingly diverse variety of “hyperactive” ALAS variants can be created and isolated by simply mutagenizing non-conserved loop residues [36]. In some cases the rate of loop opening is increased to the point where it no longer limits the overall reaction rate, in which case decay of the quinonoid intermediate becomes the slowest step [33,36].

As of this writing there is no definitive biochemical explanation as to why a relatively modest conformation change associated with product release should determine the rate of porphyrin biosynthesis in animals, but it seems likely that this situation has evolved to allow for some form of regulation of activity that still awaits discovery. One possibility would be allosteric control of the ALAS conformer equilibrium by a small molecule such as iron or heme. The animal enzyme contains a C-terminal extension with a conspicuously conserved C-X-X-C motif that might form a binding site for a small allosteric effector such as heme or iron. Mutations associated with the disease X-linked dominant protoporphyria, which is characterized by increased ALAS activity and accumulation of porphyrins, have been observed in this region of the enzyme [37]. Another possibility is that functional interaction with one or more other enzymes, such as succinyl-Coenzyme A synthetase [38], modulates loop mobility and therefore enzyme activity. It is even possible that the conformer equilibrium is post-translationally regulated by phosphorylation or nitrosylation, although there is no experimental data to support this possibility at this time. It is important to unravel the mystery behind the deliberately slow conformational control of ALAS activity, because it would represent an important step forward in our understanding of the regulation of heme biosynthesis and aerobic metabolism.

4. Recent advances in understanding ALAS structure and mechanism

Several conserved active site residues have recently been characterized by site-directed mutagenesis [14,16,39]. These fall into the three broad categories defined here and in Fig. 4,

namely: residues forming a hydrogen bonding bridge between the PLP ring nitrogen and the carbonyl derived from succinyl-Coenzyme A; residues donating a hydrogen bond to the PLP phenolate oxygen atom; and residues that bind and orient the carboxylate tail of succinyl-Coenzyme A.

Of the four residues spanning the electron sinks of the cofactor ring nitrogen and the substrate carbonyl, only mutation to murine erythroid ALAS position D279 has produced variants that are not refractory to purification.³ Studies with D279 verified that this residue enhances the electron withdrawing properties of the cofactor, and that a carboxylic amino acid at this position is essential for activity to be observed [40]. Of the other three residues, four different mutations each at positions N184 and S209 resulted in proteins localized in inclusion bodies, none of which were refoldable in amounts necessary for characterization, and three different mutations at H207 resulted in proteins so unstable that overproduction of active enzyme was not possible.⁴ It therefore seems likely that the integrity of this hydrogen bond network is crucial for proper enzyme folding and stability, in addition to activity.

The functional role of the phenoxy-like anion in PLP biochemistry is, generally speaking, two-fold. It promotes catalysis by stabilizing the electron withdrawing iminium cation, while simultaneously serving as an effective enzyme binding determinant that facilitates stereoelectronic control of amino acid α -carbon activity [41,42]. The phenoxy-like anion of ALAS-bound PLP acts as a hydrogen bond acceptor for the side chains of two residues, H282 and S254, with a subtle but important difference between the two. While H282 donates a hydrogen bond to PLP in both the open and closed enzyme conformations, S254 only comes within hydrogen bonding distance of the cofactor in the closed conformation [14,39]. As one might expect, mutation of H282 to alanine has multiple effects on the enzyme kinetic and cofactor properties, including losses in catalytic efficiencies for substrates ranging from two-to-three orders of magnitude, and alterations in the protonation state and microenvironment about the PLP cofactor as seen in absorbance, fluorescence and circular dichroism spectra. In particular the circular dichroism spectra of the holoenzymes and external aldimines with glycine or ALA indicate that in H282A ALAS the cofactor does not undergo the same $\sim 15^\circ$ reorientation observed in the wild-type enzyme [13,39].

The side chain of S254 oscillates between hydrogen bonding to the amide linkage with M255 in the open conformation, and the cofactor phenoxy-like anion in the closed conformation [13,14]. This is with the interesting caveat that the two oxygen atoms in question are each slightly closer to the succinyl-Coenzyme A sulfur atom than to each other. These structural features suggest that S254 might couple the enzyme conformational change with cofactor and substrate binding and orientation. This supposition is validated by kinetic assays with the S254A mutant, in which both the K_m^{SCoA} and k_{cat} are increased, by 32- and 3-fold, respectively [14]. These and other supporting kinetic and spectroscopic data were interpreted as reflecting a loss in succinyl-Coenzyme A binding affinity and optimal orientation relative to the cofactor, along with lessened stability of the closed conformation, the latter resulting in a 3-fold increase in k_{cat} due to faster opening of the active site loop following catalysis.

ALAS forms multiple hydrogen bonds to the carboxyl tail of succinyl-Coenzyme A, the most prominent of which are donated by R85 and T430 (Fig. 6). As noted previously, T430 is located at the apex of the active site loop and forms an unusually strong hydrogen bond with the substrate carboxyl, wherein the hydrogen atom moves freely between the two

⁴Lendrihas, Zhang, and Ferreira, unpublished observations.

oxygen atoms. This strong interaction is further enhanced by R85, which donates hydrogen bonds to each of the oxygen atoms involved in the low barrier hydrogen bond.

Acetyl-Coenzyme A is not a substrate for ALAS, and alternative four carbon Coenzyme A esters are very poor substrates, suggesting that selective alteration of R85 or T430 might lead to enzymes with altered Coenzyme A substrate specificities [16,43]. This was recently shown to be the case, although the double mutant R85L/T430V resulted in a reduction of wild-type activity by approximately 99%. Remarkably, the conservative R85K mutation resulted in an enzyme that switched specificity from succinyl-Coenzyme A to butyryl-Coenzyme A, without loss of activity.

4.1. Targeting the active site loop to generate hyperactive ALAS variants

Screening a bacterial library of ALAS variants for hyperactivity is facilitated by the observation that porphyrin fluorescence is proportional to ALAS activity [36]. By targeting non-conserved residues in the active site loop for directed-evolution based mutagenesis nine hyperactive ALAS variants were generated, isolated, and characterized. In general, catalytic efficiencies for succinyl-Coenzyme A were increased by about two orders of magnitude, while those for glycine were increased by approximately one. In three of the variants the rate of opening of the active site loop to release products was specifically accelerated to the point where it no longer limited the overall reaction rate. It was also noteworthy that all of the other observable reaction steps were also accelerated, albeit to lesser extents. This generality suggests that active site loop movements may be an integral component of multiple reaction steps, including decarboxylation and protonation of the enol-quinonoid intermediate. In other words individual reaction steps may occur while the loop is in motion, rather than occurring only once the “closed” conformation is fully realized.

4.2. New twists to ALAS function

In at least some *Streptomyces* species, including *S. nodosus* and *S. aizunensis* NRRL-B-11277, a novel ALAS has been recently discovered that generates ALA specifically for use in biosynthesis of the 2-amino-3-hydroxycyclopent-2-enone moiety of a variety of structurally related antimicrobial compounds, such as asukamycin and manumycin [44–46]. This latter compound also inhibits Ras farnesyltransferase, and is commonly used in anticancer research [47].

4.3. ALAS and clinical significance

The prominence of ALAS in day-to-day metabolism translates into a relatively low tolerance for genetic alterations, but a variety of mutations have been associated with human disease [13,37,48,49]. Interestingly, these cause one of two very different diseases, depending upon whether ALAS activity is decreased or increased. Decreases in activity are associated with X-linked sideroblastic anemia, which is characterized by pathological accumulation of *iron* in the mitochondria of erythroid precursors [49]. On the other hand, mutations at the C-terminus of ALAS can result in enhanced activity and X-linked dominant protoporphyria, which is characterized by accumulation of *protoporphyrin* in plasma and red blood cells [37]. These very different diseases emphasize the cellular requirement for tight coupling of ALAS controlled protoporphyrin synthesis with iron transport during heme biosynthesis.

5. Summary, prospects, and outlook

The current model for ALAS catalysis is summarized as follows. The holoenzyme is in conformational equilibrium between “open” and “closed” states, as seen in the crystal structures and depicted in Fig. 5. Collision complex binding of glycine is rapid but weak,

and has little effect on the conformer equilibrium. Subsequent binding of succinyl-Coenzyme A leads to a dramatic shift in conformer equilibrium favoring the closed state. The primary succinyl-Coenzyme A binding determinants leading to the collapse of the enzyme around the Michaelis complex are clustered about the adenine ring and the succinyl carboxyl. The linkage between these determinants is cleaved during the reaction chemistry, which is relatively rapid, facilitating a slow, rate-determining return to the open conformation wherein the reactions products are rapidly released.

One interesting and outstanding question is the extent to which the ALAS enzyme-substrate complex reacts as if it were a unimolecular complex. Obviously, it is assumed to be unimolecular when analyzed using Michaelis–Menten kinetics, but what we really mean here is the extent to which protein dynamics might be inseparable from active site chemistry [50]. For instance, are the observable chemical steps coincident with observable motions of the active site loop? A fluorescence tag approach similar to that taken for DNA polymerase [51,52] might shed further light on this possibility, as well as helping to better understand the translation of succinyl-Coenzyme A binding energy into a change in conformational distribution.

Hyperactive ALAS variants similar to those generated *in vitro* [36] or *in vivo* [37] may find utility in producing local accumulation of protoporphyrin IX during photodynamic therapies [53,54]. This approach may find particular utility in conjunction with tumor immunological approaches, potentially leading to anti-tumor immunity [55,56].

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Fig. 1.
ALAS directly couples heme biosynthesis to aerobic respiration.

Fig. 2. Dimeric structure of ALAS. The PLP external aldimine with glycine is depicted with *yellow* carbons, and residues over 20 down the active site cleft, which is defined by the extensive binding site for succinyl-Coenzyme A (*white carbons*).

Fig. 3.

ALAS chemical mechanism. See text for details.

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Fig. 4. ALAS active site model for the external aldimine to the 1-amino-2-ketoadipate intermediate. A tetrad of residues form a hydrogen bonding network that links the electron sinks of the PLP ring nitrogen to the intermediate ketone functional group. These hydrogen bonds are shown in *yellow*. Hydrogen bonds donated to the PLP phenolate are in *pink*, while those to the carboxylate tail are in *light green*. At this point in the reaction cycle the α -carbon carboxyl is lost and then replaced by a proton donated from K313 to form the product bound external aldimine. The misalignment of the α -carbon carboxyl for decarboxylation directly into the ring is resolved by H207 acting as an acid catalyst for decarboxylation through the ketone to form an enol that is in equilibrium with a quinonoid intermediate.

Fig. 5.
Conformational change of the active site loop.

Scheme 1.

Kinetic mechanism of murine erythroid ALAS at 30 °C. The steps corresponding to chemistry in Fig. 3 are denoted in blue, while the conformational state is indicated in red or green.

Fig. 6. Binding interactions with succinyl-Coenzyme A stabilize the closed conformation. ALAS forms multiple strong and specific binding interactions with both the adenine ring and the succinyl tail of the substrate, causing the two enzyme domains associated with these binding sites to collapse into a “closed” conformation upon binding the substrate. The active site loop is in *semi-transparent red*, and hydrogen bonds donated by residues on the adjacent subunit of the homodimer are depicted with *dark blue* carbons.