Active Site of 5-Aminolevulinate Synthase Resides at the Subunit Interface. Evidence from *in Vivo* Heterodimer Formation†

Dongwei Tan and Gloria C. Ferreira*,‡

Department of Biochemistry and Molecular Biology, College of Medicine, Institute for Biomolecular Science, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612

Received December 11, 1995; Revised Manuscript Received March 21, 1996⊗

ABSTRACT: 5-Aminolevulinate synthase (EC 2.3.1.37) is the first enzyme in the heme biosynthetic pathway of animals, fungi and some bacteria. It functions as a homodimer and requires pyridoxal 5′-phosphate as an essential cofactor. In mouse erythroid 5-aminolevulinate synthase, lysine 313 has been identified as the residue involved in the Schiff base linkage with pyridoxal 5′-phosphate [Ferreira, G. C., et al. (1993) *Protein Sci. 2*, 1959−1965], while arginine 149, a conserved residue among all known 5-aminolevulinate synthase sequences, is essential for function [Gong & Ferreira (1995) *Biochemistry 34*, 1678−1685]. To determine whether each subunit contains an independent active site (i.e., intrasubunit arrangement) or whether the active site resides at the subunit interface (i.e., intersubunit arrangement), *in vivo* complementation studies were used to generate heterodimers from site-directed, catalytically inactive mouse 5-aminolevulinate synthase mutants. When R149A and K313A mutants were co-expressed in a *hem A*$^-$ *Escherichia coli* strain, which can only grow in the presence of 5-aminolevulinate or when it is transformed with an active 5-aminolevulinate synthase expression plasmid, the *hem A*$^-$ *E. coli* strain acquired heme prototrophy. The purified K313A/R149A heterodimer mixture exhibited K_m values for the substrates similar to those of the wild-type enzyme and approximately 26% of the wild-type enzyme activity which is in agreement with the expected 25% value for the K313A/R149A coexpression system. In addition, DNA sequencing of four *Saccharomyces cerevisiae* 5-aminolevulinate synthase mutants, which lack ALAS activity but exhibit enzymatic complementation, revealed that mutant G101 with mutations N157Y and N162S can complement mutant G220 with mutation T452R, and mutant G205 with mutation C145R can complement mutant Ole3 with mutation G344C. Taken together, these results provide conclusive evidence that the 5-aminolevulinate synthase active site is located at the subunit interface and contains catalytically essential residues from the two subunits.

5-Aminolevulinate synthase (ALAS,[1] EC 2.3.1.37) catalyzes the first reaction in the heme biosynthetic pathway, glycine + succinyl-CoA → ALA + CoASH + CO_2, in nonplant eukaryotes and some prokaryotes (Jordan, 1991; Ferreira & Gong, 1995). In animals, there are two separate genes that encode two different ALAS isoforms, one for a housekeeping form and the other for an erythroid-specific form (Riddle et al., 1989). Further, the gene for human erythroid ALAS has been localized on the X-chromosome (Bishop et al., 1990; Cox et al., 1990), whereas the gene for human housekeeping ALAS is located on chromosome 3 (Sutherland et al., 1988; Bishop et al., 1990). Interestingly, two separate ALAS genes, *hemA* and *hemT*, have also been identified in *Rhodobacter spheroides* (Neidle & Kaplan, 1993). The *hemA* and *hemT gene* products are homologous to other characterized ALASs. It was found that *hemA* transcripts were expressed at higher levels under photosynthetic conditions than those under aerobic conditions, and *hemT* was expressed in a mutant strain in which the *hemA* gene had been inactivated, but not in wild-type cells under physiological growth conditions (Neidle & Kaplan, 1993). Both in animals and *R. spheroides*, the two ALAS genes, respective transcripts and enzymes, seem to be under distinct control mechanisms (Neidle & Kaplan, 1993; Ferreira & Gong, 1995).

ALAS functions as a homodimer and requires pyridoxal 5′-phosphate (PLP) as an essential cofactor. In mouse erythroid ALAS, lysine 313 (K313) appears to be the residue involved in the binding of the PLP cofactor through a Schiff base linkage between its ϵ-amino group and the PLP cofactor aldehyde group. In addition, mutagenesis of this residue to alanine or histidine abolishes the enzyme activity (Ferreira et al., 1993). Recently, arginine 149 (R149) in mouse erythroid ALAS was also shown to be catalytically essential (Gong & Ferreira, 1995). These two residues are conserved in all known ALASs. Two active site arrangements can be proposed to explain the dimeric nature of ALAS. One is the intersubunit arrangement, in which the active sites are located at the interface between subunits; the other is the intrasubunit arrangement, in which each active site is wholly contained within a subunit.

To distinguish between the possible active site arrangements of ALAS (i.e., inter- and intrasubunit), we developed *in vivo* complementation strategies in which two inactive

† This work was supported by the National Science Foundation (MCB-9206574) (to G.C.F.).

* Author to whom correspondence should be addressed. Telephone: (813) 974-5797. Fax: (813) 974-5798. E-mail: gferreir@com1.med.usf.edu.

‡ Recipient of a NSF Young Investigator Award (MCB-9257656).

⊗ Abstract published in *Advance ACS Abstracts*, June 15, 1996.

[1] Abbreviations: ALA, 5-aminolevulinate; ALAS, 5-aminolevulinate synthase; PLP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

S0006-2960(95)02918-7 CCC: $12.00

mouse ALAS mutants (K313A and R149A) were coexpressed in *Escherichia coli*; we demonstrated that ALAS is an intersubunit dimer with its active sites at the subunit interface. In addition, DNA sequencing of yeast *Saccharomyces cerevisiae* ALAS mutants, which previously were reported not to have enzymatic activity but to exhibit complementation and yet did not revert to the wild-type as verified by meiotic analysis (Urban-Grimal & Labbe-Bois, 1981), showed that mutants with mutation(s) close to the N-terminus of the enzyme could complement mutants with mutation(s) close to the C-terminus, which can be clearly interpreted by the proposed intersubunit arrangement of ALAS active sites.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs and were used according to the supplier's instructions. GeneClean II kit was a product of Bio 101 Inc. Acrylamide and gel reagents were purchased from Bio-Rad. Sequenase and sequencing kit were from U. S. Biochemical Corp. [α-^{35}S]dATP was from Dupont/NEN Research Products. The bicinchoninic acid protein assay reagents were obtained from Pierce Chemical Co. The Superose 6 gel filtration column was from Pharmacia. DEAE-Sephacel was obtained from Sigma and Ultrogel ACA-44 was from IBF. Ni-NTA agarose was purchased from Qiagen. The oligonucleotide primers were synthesized in DNA Synthesis Core Laboratory, University of Florida or GIBCO BRL. The Molecular Weight Markers kit for gel filtration chromatography was obtained from Sigma. All other chemicals were of the highest purity available. pACYC184 was from New England Biolabs. Yeast *S. cerevisiae* genomic DNA from wild-type and ALAS mutant strains were given generously by Dr. Rosine Labbe-Bois (Institut Jacques Monod, Paris, France). The *E. coli* strain HU227 (Li et al., 1989) was a gift from Dr. C. S. Russell (City University of New York).

Methods

Construction of Plasmid pDT4. The pDT4 plasmid was constructed from pGF27 (Ferreira et al., 1993) and contains a full length K313A mouse ALAS mutant, in which lysine-313 was mutated to alanine. A DNA fragment encoding the alkaline phosphatase (*phoA*) promoter and the full length K313A ALAS mutant was retrieved from pGF27 upon digestion with *Cla*I and *Nru*I. Then the fragment was ligated in pACYC184 (Chang & Cohen, 1978; Rose, 1988), previously digested with *Cla*I and *Nru*I. An aliquot of the ligation reaction was used to transform *E. coli* strain DH5α. This yielded a K313A ALAS mutant overexpression plasmid with a chloramphenicol resistance gene.

Construction of Histidine-Tagged Wild-Type (pDT6) and K313A Mutant (pDT8) Mouse ALAS Overexpression Plasmids. The pDT6 plasmid, constructed from pGF23 (Ferreira & Dailey, 1993), contains a full-length mouse ALAS wild-type sequence with a N-terminal extension consisting of five histidine residues. The cDNA encoding histidine-tagged wild-type ALAS was generated by using the full-length wild-type ALAS-containing plasmid pGF23 as a template for the polymerase chain reaction (PCR) on a MJ Research MiniCycler. The 5′-end primer, 5′-ATA TGT CGA CTC ACC ACC ACC ACC ACA TTG TGC AGA GGG CAG CT-3′, encoded the first six N-terminal amino acids of ALAS, five histidine residues, and a *Sal*I site. The 3′-end primer, 5′-GCA GCT GGA TCC TTA AGC ATA GGT GGT AAC AT-3′, encoded the last five amino acids, a stop codon, and a *Bam*HI site. The reaction mixture (100 μL) contained 1.5 ng of DNA template, 20 nmol of triphosphate dideoxynucleotides, 50 pmol of each primer, 2.5 units of Taq DNA polymerase, and reaction buffer (Boehringer Mannheim). A total of 25 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 4 min was followed by an extension of 6 min at 72 °C. The PCR-amplified DNA fragment was digested with *Sal*I and *Kpn*I and subcloned into pGF23 (Ferreira & Dailey, 1993), previously digested with *Sal*I and *Kpn*I. This yielded a histidine-tagged wild-type mouse ALAS overexpression plasmid. The PCR-amplified mouse ALAS fragment was verified by DNA sequencing according to the dideoxy chain termination method (Sanger et al., 1977). The pDT8 plasmid was constructed from pDT6 and pDT4 and contains a full-length mouse ALAS K313A mutant with an N-terminal extension consisting of five histidine residues. A DNA fragment encoding the partial histidine-tagged wild-type mouse ALAS was retrieved from pDT6 upon digestion with *Sal*I and *Kpn*I. Then the fragment was ligated in pDT4, previously digested with *Sal*I and *Kpn*I. This yielded a histidine-tagged K313A mouse ALAS mutant overexpression plasmid.

Transformation of E. coli Cells. E. coli HU227 or DH5α cells transformed with pJGR149A (Gong & Ferreira, 1995), pGF23 (Ferreira & Dailey, 1993), or pDT6 were grown in LB medium (Sambrook et al., 1989) containing 50 μg/mL ampicillin, with or without 10 μg/mL ALA, respectively. HU227 or DH5α cells transformed with pDT4 or pDT8 were grown in LB medium containing 35 μg/mL chloramphenicol, with or without 10 μg/mL ALA, respectively. HU227 or DH5 cells cotransformed with pJGR149A and pDT4 or pDT8 were grown in LB medium containing 50 μg/mL ampicillin and 35 μg/mL chloramphenicol.

Expression and Purification of Wild-Type and Mutant Mouse ALASs. Wild-type and mutant enzymes were purified from bacterial overproducing cells containing ALAS-encoding cDNAs under the control of the alkaline phosphatase (*phoA*) promoter. *E. coli* strain DH5α cells harboring the ALAS expression plasmid(s) were grown in MOPS (Ferreira & Dailey, 1993) medium containing 50 μg/mL ampicillin, 35 μg/mL chloramphenicol, or both where appropriate, at 37 °C with shaking at 225 rpm. Purification of wild-type and mutant enzymes was accomplished using a 4 L culture as the starting material, as previously described for the wild-type enzyme (Ferreira & Dailey, 1993) except for one modification. The supernatant, resulting from the centrifugation of the ALAS overproducing cell lysate, was fractionated by addition of saturated ammonium sulfate to a concentration of 20% saturation. After 10 min of stirring at 4 °C, the solution was centrifuged at 15000*g* for 15 min. The resulting supernatant was further fractionated with ammonium sulfate to a final concentration of 40% saturation. Purification of histidine-tagged wild-type mouse ALAS and histidine-tagged K313A/R149A heterodimer mixture was accomplished using nickel chelate agarose chromatography. *E. coli* strain DH5α cells harboring the histidine-tagged ALAS expression plasmid pDT6, or pDT8 and pJGR149A together, were harvested

and resuspended in 20 mM tricine buffer, pH 8.0, containing 20 μM PLP, 5 mM β-mercaptoethanol, 10% glycerol, 100 μM PMSF, and 1 μg/mL each of the three protease inhibitors, aprotinin, leupeptin, and pepstatin. The cells were lysed through French press at 10 000 psi, and the lysate was centrifuged at 15000*g* for 10 min. Then the supernatant was loaded onto a nickel-NTA agarose (Qiagen) column (1.5 × 10 cm). The column was washed with 4−5 column volumes of 20 mM tricine buffer, pH 8.0, containing 20 μM PLP, 5 mM β-mercaptoethanol, and 10% glycerol, and then with 4−5 column volumes of the same tricine buffer containing 0.5 M sodium chloride. Subsequently, the column was washed with 4−5 column volumes of 1 mM imidazole buffer, pH 7.2, containing 20 μM PLP, 5 mM β-mercaptoethanol, and 10% glycerol. Finally, the protein was eluted with 20 mM imidazole buffer, pH 7.2, containing 20 μM PLP, 5 mM β-mercaptoethanol, and 10% glycerol. The collected protein fractions were examined by 15% SDS−PAGE (Laemmli, 1970) and activity assay (Hunter & Ferreira, 1995).

Molecular Mass Determination of the Mouse ALAS K313A/R149A Heterodimer Mixture by Gel Filtration Chromatography. The native molecular mass of the purified mouse ALAS K313A/R149A heterodimer mixture was determined by gel filtration chromatography on Superose 6 gel (Pharmacia). The Superose 6 gel filtration column, which was connected to a Perkin Elmer HPLC system, was equilibrated with 20 mM imidazole buffer, pH 7.2, containing 20 μM PLP at a flow rate of 0.5 mL/min. The gel filtration molecular weight standards (Sigma) were dissolved in the same buffer and eluted from the column at a flow rate 0.5 mL/min. The calibration curve was obtained by plotting logarithm (molecular weight) versus elution volume for each molecular weight standard. A purified mouse ALAS $(His)_5$-K313A/R149A heterodimer mixture sample was loaded onto the column, and the run was conducted following the same procedure as that of the molecular weight standards. The molecular mass of the heterodimer mixture was calculated using the equation derived from the calibration curve.

SDS−PAGE, Protein Concentration, and Kinetic Studies. SDS−PAGE was performed as described by Laemmli (1970); 15% acrylamide and 1.5-mm thick gels were used. Aliquots of the protein samples (100 μL) were heated for 3 min at 95 °C in SDS−mercaptoethanol loading buffer, and the proteins were visualized upon Coomassie Brilliant Blue (R-250) staining. Before the determination of protein concentration, acetone was used to precipitate the protein in the samples and the standards. Specifically, 1 mL of acetone was added to each test tube containing either the protein sample or the standard. After 10 min incubation on ice, samples and standards were centrifuged at 10000*g* for 4 min. The pellets were resuspended in 50 μL of H_2O. Then, the protein concentrations were determined by the bicinchoninic acid assay according to the manufacturer's instructions. ALAS enzyme activity was measured using a continuous spectrophotometric assay (Hunter & Ferreira, 1995). Lineweaver−Burk double-reciprocal plots were employed to obtain apparent K_m and V_{max} values. Apparent K_m and V_{max} were determined for glycine and succinyl-CoA at constant concentrations of 50 μM for succinyl-CoA and 300 mM for glycine, respectively.

PLP Determination. Before the determination of the stoichiometric ratios of PLP in the different ALAS variants, acetone was used to precipitate the protein in the samples, as described in the protein concentration determination method (see above). After precipitation, the pellets were washed with 1 mL of acetone before resuspended in 10 μL of H_2O. Then, PLP was determined by the fluorimetric method of Adams (1979).

Construction of Yeast ALAS DNA Sequencing Templates and DNA Sequencing. The DNA fragments encoding the mutant yeast ALAS proteins were subcloned into pBluescript SK+ using PCR and the mutant yeast genomic DNA (Urban-Grimal & Labbe-Bois, 1981) as DNA templates. The 5′-end primer, 5′-CGC GGA TCC ATG CAA CGC TCC ATT TT, contained the sequence for the first six N-terminal amino acids and a *Bam*HI site. The 3′-end primer, 5′-CCG GAA TTC TTA CTG CTT GAT ACC AC-3′, contained the sequence for the five C-terminal amino acids, a stop codon, and a *Eco*RI site. The reaction mixture (100 μL) contained 1 μg of DNA template, 20 nmol of triphosphate dideoxynucleotides, 50 pmol of each primer, 2.5 units of Taq DNA polymerase, and reaction buffer (Boehringer Mannheim). A total of 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 74 °C for 3 min was followed by an extension of 10 min at 74 °C. The PCR- amplified DNA fragments were purified and digested with *Bam*HI and *Eco*RI. Then the fragments were ligated into pBluescript SK+, previously digested with *Bam*HI and *Eco*RI. Aliquots of the different ligation reactions were used to transform *E. coli* strain DH5α, and the mutant yeast ALAS- containing plasmids were selected in ampicillin-resistant transformants containing the correct size insert. To identify the mutations in the ALAS-encoding fragments and to minimize the probability of occurrence of PCR errors, 8−10 transformants generated from the same ligation reaction were used to make DNA sequencing templates according to the alkaline lysis method (Sambrook et al., 1989). Subsequently, the independently made DNA templates were mixed and used in DNA sequencing, as described in the dideoxynucleotide chain termination method (Sanger et al., 1977). Manual (as described above) and automated (DNA Sequencing Core Laboratory, University of Florida) sequencing was used to determine the DNA sequence of the mutant and yeast ALAS genes in both orientations.

RESULTS

In Vivo Complementation by Two Inactive Mouse ALAS Mutants Cotransformed in E. coli HU227 Cells. Protein sequence alignments indicate that murine erythroid ALAS lysine 313 and arginine 149 are conserved residues in all known ALASs (Ferreira & Gong, 1995) (Figure 1). Mutagenesis experiments suggest that these two residues may play important role(s) in the active site of ALAS. To distinguish between the two possibilities for the active site arrangement of the ALAS dimer (i.e., inter- and intrasubunit) (Figure 2), we followed an experimental approach involving the use of two inactive ALAS site-directed mutants (K313A and R149A) and an efficient biological selection system (*E. coli* HU227). Specifically, *hemA*$^-$ *E. coli* strain HU227 can only grow in a medium supplemented with ALA or when transformed with plasmid(s) which express a functional ALAS. Therefore, the rescue of HU227 cells growth in a non-ALA supplemented medium indicates the production of an active ALAS.

	149										313														
Mouse erythroid	H	G	A	G	A	G	G	T	**R**	N	I	D	I	I	S	G	T	L	G	**K**	A	F	G	C	V
Human housekeeping	H	G	A	G	A	G	G	T	**R**	N	I	D	I	I	S	G	T	L	G	**K**	A	F	G	C	V
Human erythroid	H	G	A	G	A	G	G	T	**R**	N	I	D	I	I	S	G	T	L	G	**K**	A	F	G	C	V
Rat housekeeping	H	G	A	G	A	G	G	T	**R**	N	I	D	I	I	S	G	T	L	G	**K**	A	F	G	C	V
Rat erythroid	H	G	A	G	A	G	G	T	**R**	N	I	D	I	I	S	G	T	L	G	**K**	A	F	G	C	V
Chicken housekeeping	H	G	A	G	A	G	G	T	**R**	N	I	D	I	I	S	G	T	L	G	**K**	A	F	A	C	V
Chicken erythroid	H	G	L	G	A	G	G	T	**R**	N	I	D	V	V	S	G	T	L	G	**K**	A	L	G	A	V
Saccharomyces cerevisiae hem1	Y	G	C	G	A	G	G	T	**R**	N	I	D	M	I	T	G	T	L	G	**K**	S	F	G	S	V
Aspergillus nidulans	Y	G	A	G	A	G	G	T	**R**	N	I	N	I	I	T	G	T	L	G	**K**	A	Y	G	C	V
Paracocus denitrificans	T	G	A	G	S	G	G	T	**R**	N	I	D	I	F	N	G	T	L	A	**K**	A	F	G	V	F
Rhodobacter spheroides hemT	S	G	A	G	T	G	G	T	**R**	N	I	T	I	I	E	G	T	L	A	**K**	A	F	G	V	M
Rhodobacter spheroides hemA	T	G	A	G	S	G	G	T	**R**	N	I	D	I	I	N	G	T	L	G	**K**	A	Y	G	V	F
Rhodobacter capsulatus	V	G	A	G	S	G	G	T	**R**	N	I	D	I	F	N	G	T	L	A	**K**	A	Y	G	V	F
Agrobacterium radiobacter	C	G	A	G	A	G	G	T	**R**	N	I	T	I	I	E	G	T	L	G	**K**	A	F	G	V	M
Bradyrhizobium japonicum	V	G	T	G	A	G	G	T	**R**	N	I	D	I	L	E	G	T	L	A	**K**	A	F	G	C	L

FIGURE 1: Alignment of all known ALAS sequences around mouse erythroid ALAS arginine 149 and lysine 313 residues. Nonhomologous amino acids are underlined.

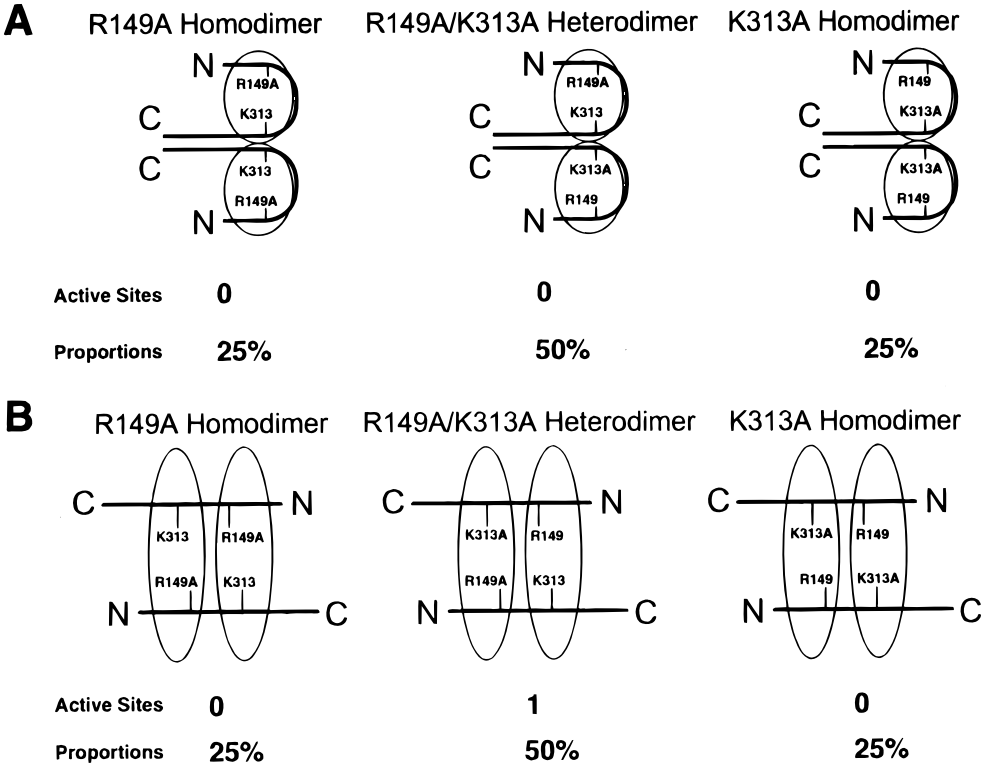

FIGURE 2: Schematic presentation of two possible active site arrangements for ALAS and the expected dimer combinations after *in vivo* heterodimer formation. Possible intrasubunit and intersubunit active site arrangements for ALAS. Each active site, which contains lysine 313 and arginine 149 residue, is ellipsed. Each intrasubunit active site contains lysine and arginine residues both from a single polypeptide chain while an intersubunit active site requires participation of lysine and arginine residues from both subunits. In both cases, there are two active sites per dimer. (A) Inactive mutant homodimers and inactive mutant heterodimers if ALAS follows an intrasubunit active site arrangement. (B) Inactive mutant homodimers and partially active mutant heterodimers if ALAS follows an intersubunit active site arrangement. The expected specific activity of the mixed intersubunit dimer population is 50% × 50% = 25% of that of the wild-type. In both A and B, equal amounts of two mutant proteins and random interaction are assumed in the *in vivo* heterodimer formation.

Competent HU227 cells were transformed with pJGR149A, pDT4 (Figure 3), or pJGR149A and pDT4 together and screened for resistance to ampicillin, chloramphenicol, or ampicillin and chloramphenicol. pJGR149A and pDT4 separately cannot produce a functional ALAS. If the active site of mouse ALAS involves lysine 313 and arginine 149 from different polypeptide chains, then two subunits of the K313A/R149A heterodimer will complement each other and produce a partially functional ALAS which will contain only one active site (Figure 2); the HU227 cells with these two plasmids will grow in LB medium with ampicillin and chloramphenicol but no ALA, otherwise they will require ALA.

Table 1 shows the results of transformation of HU227 cells with inactive mouse ALAS mutant expression plasmids (either isolated or in combination) and with the wild-type mouse ALAS expression plasmid as a positive control. As expected, when HU227 was transformed with either the R149A or K313A mutant, no functional ALAS was produced, and no colonies could be observed on the LB plates

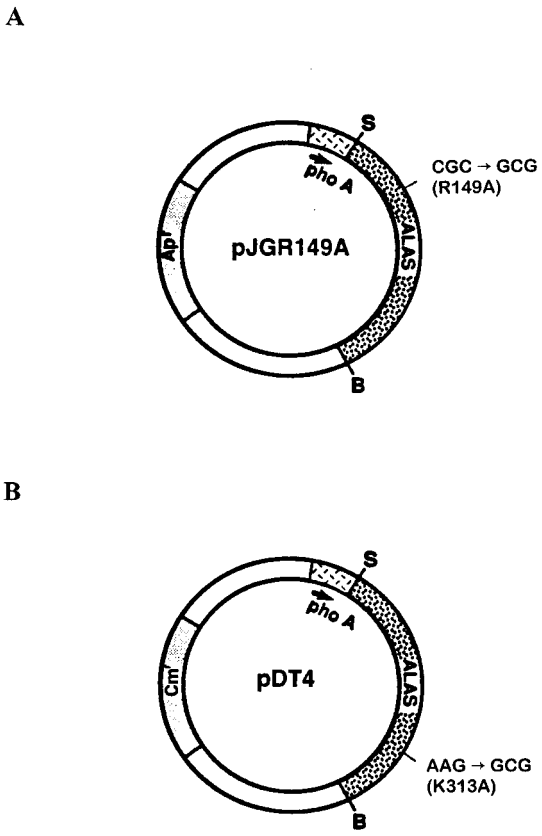

FIGURE 3: Plasmids used for in vivo coexpression of mutant mouse ALASs. (A) pJGR149A, a cDNA encoding ALAS, in which arginine 149 is mutated to alanine, is under the control of a *phoA* promoter and possesses an ampicillin resistance selectable marker. (B) pDT4, a cDNA encoding ALAS, in which lysine 313 is mutated to alanine, is under the control of a *phoA* promoter and possesses a chloramphenicol resistance selectable marker. ALAS heterodimer/homodimer mixture containing the R149A and K313A mutants were purified from *E. coli* DH5 cells carrying pJGR149A and pDT4. *phoA*, alkaline phosphatase promoter; *Ap*^{r}, ampicillin resistance gene; *Cm*^{r}, chloramphenicol resistance gene; S, *Sal*I; B, *Bam*HI.

Table 1: HU227 Transformation by Different Plasmids

plasmid(s)	medium	growth
none	LB + Amp[a] + Cm[b]	−
pJGR149A (R149A mutant)	LB + Amp	−
pDT4 (K313A mutant)	LB + Cm	−
pJGR149A + pDT4	LB + Amp + Cm	+
pGF23[c]	LB + Amp	+

[a] Amp, ampicillin. [b] Cm, chloramphenicol. [c] pGF23 is a wild-type ALAS overexpression plasmid.

without ALA. However, when HU227 was cotransformed with pJGR149A and pDT4, there were many colonies on the LB plates with ampicillin and chloramphenicol, even without the supplement of ALA. This is the first evidence that ALAS has an intersubunit active site arrangement and that the active site consists of catalytically essential residues from different subunits.

Kinetic Parameters of In Vivo Generated Homo- and Heterodimeric Mutant Mouse ALASs. To investigate further the active site arrangement of ALAS, we next examined the enzymatic activities of mutant enzymes expressed in *E. coli* DH5α cells. The mutant proteins were overexpressed at the same level as that of the wild-type and purified to homogeneity. There was no measurable activity detected in these two mutants using a standard assay. We also purified the active mouse ALAS heterodimer/homodimer mixture from the DH5α cells cotransformed with two mutant plasmids (i.e., pJGR149A and pDT4). The apparent K_m values for glycine and succinyl-CoA and V_{max} of the dimer mixture were determined and compared to those of the wild-type. The results of these determinations are summarized in Table 2. The apparent K_m values for the dimer mixture are 6.95 mM and 1.52 μM for glycine and succinyl-CoA, respectively, which are comparable to the values of the wild-type enzyme. This suggests that there was regeneration of an unmodified ("native") active site in the heterodimer through complementation of two inactive ALAS mutants. Further, the apparent V_{max} of the dimer mixture corresponded to 26.4% of that of the wild-type enzyme.

Molecular Mass of the Mouse ALAS K313A/R149A Heterodimer Mixture As Determined by Gel Filtration Chromatography. The molecular mass of each subunit of the recombinant mature form of mouse erythroid ALAS is ~56 kDa (Ferreira & Dailey, 1993). The molecular mass of the recombinant wild-type ALAS homodimer is 112 kDa, as determined by gel filtration chromatography. Further, R149A and K313A are also homodimers as verified by gel filtration chromatography (data not shown). To confirm the molecular mass of the native ALAS K313A/R149A heterodimer mixture, we used gel filtration chromatography. It should be emphasized that, in the context of this report, by "heterodimer" is meant a dimer of subunits with a single amino acid difference and therefore virtually identical molecular mass. The addition of a five-histidine tag at the N-terminal of the wild-type or the mutants was used to simplify the purification procedure. The N-terminal histidine tag did not alter the enzyme activity, the bound PLP/homodimer stoichiometry, and the dimerization of the wild-type ALAS (data not shown). The purified $(His)_5$-K313A/R149A heterodimer and $(His)_5$-K313A homodimer mixture was then loaded onto a Pharmacia Superose 6 gel filtration column. A chromatogram with a single peak, corresponding to a molecular mass of 119 kDa, and a subunit of 56 kDa, as verified by SDS−PAGE, indicate that the $(His)_5$-K313A/R149A dimer has two identical size subunits as in the case of the wild-type enzyme (Figure 4). In addition, the number of PLP molecules bound per dimer molecule remained the same in the heterodimer mixture as that in the wild-type (i.e., two PLP molecules bound per dimer molecule).

Identification of the Mutations in Four Yeast ALAS Mutants. Urban-Grimal and Labbe-Bois (1981) isolated a series of *S. cerevisiae* mutants lacking ALAS activity and with unusually large amount of immunodetectable protein. Significantly, the investigators showed that, through genetic crossing experiments, some of the yeast ALAS mutants could complement each other and recover 15−25% of usual enzymatic activity. Since the yeast ALAS amino acid sequence shows clear homology with other eukaryotic ALASs (Cox et al., 1991), we decided to identify the mutations, at gene level, of some of these yeast ALAS mutants. Two complementation pairs were chosen, one in which mutant G101 complements mutant G220 and the other in which mutant G205 complements mutant Ole3. After DNA sequencing, the mutations were identified as follows: N157Y and N162S in G101, T452R in G220, C145R in G205, and G344C in Ole3 (Table 3). The results obtained with the yeast ALAS mutants corroborate those for the mouse ALAS heterodimer and provide further evidence for the intersubunit arrangement of ALAS.

Table 2: Kinetic Parameters for Purified K313A/R149A and Wild-Type Mouse ALASs

recombinant protein	app K_m^{Gly} (mM)	app K_m^{Suc} (mM)	app V_{max} (nmol mg^{-1} h^{-1})	percentage of wild-type app V_{max}
WT ALAS	8.39	1.82	2.2×10^4	100
R149A			0	0
K313A			0	0
R149A/K313A (heterodimer mixture)	6.95	1.52	5.8×10^3	26.4

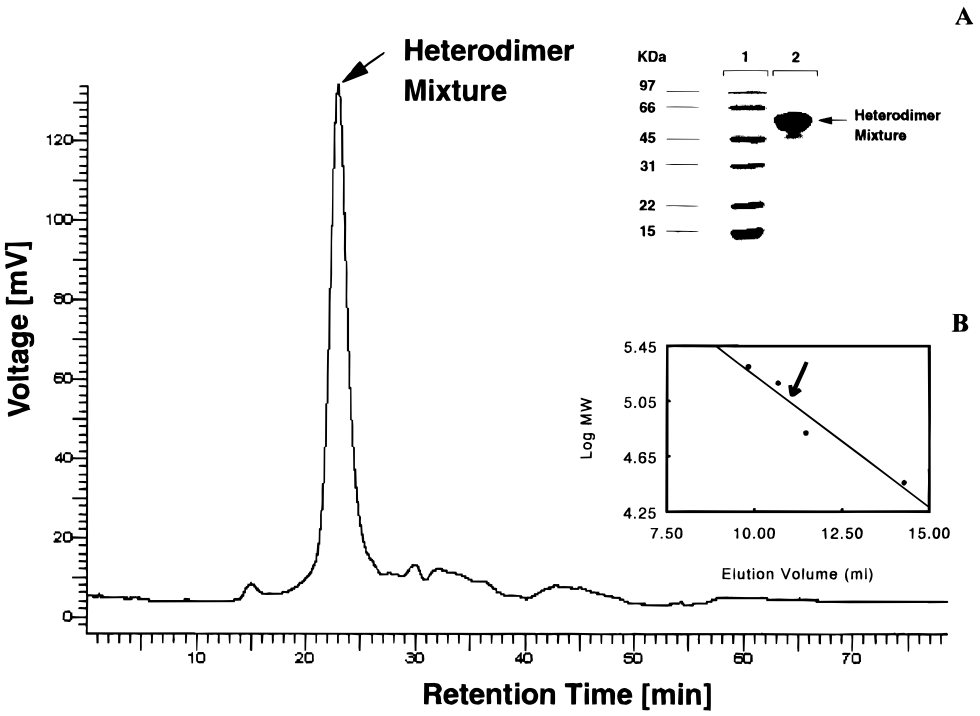

FIGURE 4: Determination of molecular mass of the $(His)_5$-K313A/R149A heterodimer (mixture) by gel filtration chromatography. $(His)_5$-K313A/R149A heterodimer mixture was applied to a Pharmacia Superose 6 gel filtration column and eluted with 20 mM imidazole, pH 7.2, containing 20 μM PLP (flow rate, 0.5 mL/min). (Insert A) SDS−PAGE of purified $(His)_5$-K313A/R149A heterodimer protein. Samples were run on 15% SDS−PAGE and detected by Coomassie Brilliant Blue (R-250) staining (see Experimental Procedures). Lane 1, standard proteins (The molecular masses are indicated on the left.). Lane 2, purified $(His)_5$-K313A/R149A heterodimer protein. (Insert B) Molecular mass calibration curve for the Pharmacia Superose 6 column. The standard proteins used were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa). The heterodimer protein is indicated by an arrow.

Table 3: Mutations Detected in Four Yeast ALAS Mutants

yeast ALAS mutant	G101	G220	G205	Ole3
mutation(s)	469 AAC→TAC 484 AAT→AGT	1354 ACG→AGG	433 TGT→CGT	1030 GGC→TGC
amino acid change(s)	Asn157→Tyr Asn162→Ser	Thr452→Arg	Cys145→Arg	Gly344→Cys

DISCUSSION

The development of an heterologous overexpression system for the murine erythroid ALAS (Ferreira & Dailey, 1993) has made it possible to address questions concerning structure and function relationships of the enzyme. Although, presently, the ALAS three-dimensional structure remains unknown, critical active site residues have been recently identified (Ferreira et al., 1993; Gong & Ferreira, 1995). Namely, the lysine 313 residue of the mouse erythroid ALAS has been shown to be involved in the Schiff base linkage with PLP, and mutagenesis of this residue to alanine, glycine, or histidine totally eliminated the enzyme activity (Ferreira et al., 1993). In addition, the arginine 149 residue has been shown to be catalytically essential and unable to tolerate any other amino acid substitutions (Gong & Ferreira, 1995).

ALAS is known to be functional as a homodimer (Ferreira & Gong, 1995); however, the location of the active site in relation to the whole enzyme and the contribution of residues from different subunits to the active site remained as unanswered questions. To obtain further insight on the structure and function relationships of ALAS, and in particular, on the topological arrangement of the enzyme's active site, we developed strategies which allowed us to propose a model for the ALAS active site, even with the unavailability of the ALAS X-ray crystallographic structure. Here, the key structural/functional roles of mouse ALAS lysine 313 and arginine 149 in catalysis were exploited to investigate the molecular arrangement of the ALAS active site. First, we observed restoration of ALAS activity when inactive K313A and R149A expression plasmids were cotransformed in a *hem A*$^-$ *E. coli* strain HU227. Secondly, following coexpression of these plasmids in *rec A*$^-$ DH5α

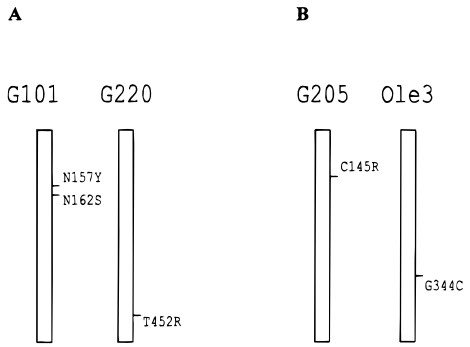

FIGURE 5: Diagrammatic representation of the mutations present in two yeast ALAS complementation pairs. (A) G101 with mutations N157Y and N162S complements G220 with mutation T452R. (B) G205 with mutation C145R complements Ole3 with mutation G344C.

cells, the purified mouse ALAS K313A/R149A heterodimer mixture exhibited approximately 26% of wild-type enzyme activity and K_m values for the substrates similar to those of the wild-type enzyme. If the two active sites of an ALAS dimer have the same amino acid composition and are equally active, equal amounts of mutant proteins coexpressed *in vivo* should result in a theoretical activity for the purified protein mixture of 25% of that of the wild-type enzyme. The experimentally determined value was 26.4%, which is close to the theoretical value, supporting the proposal that the active site of ALAS is shared by the two subunits and that therefore it resides at the subunit interface. Further, as determined by gel filtration chromatography, the molecular mass of the heterodimer mixture is 119 kDa, which is similar to that of the wild-type enzyme. Thus, it suggests that dimerization is necessary for a functional ALAS.

DNA sequencing of inactive yeast ALAS mutants supports the intersubunit active site arrangement. Amino acid sequence alignment between yeast ALAS and other eukaryotic ALASs indicates a strong homology, especially in the so-called "catalytic domain", which lies within a C- terminal region of about 400 amino acids (Cox et al., 1991). Inactive yeast ALAS mutants recovered some enzymatic activity upon complementation (Urban-Grimal & Labbe-Bois, 1981). Of significance, no wild-type recombinants could be observed in the meiotic analysis of the segregants generated from the complementation experiments, indicating that the mutations were in the ALAS structural gene. The authors suggested that the complementation could be explained by protein−protein interaction between two inactive mutant ALAS subunits (Urban-Grimal & Labbe-Bois, 1981). We chose two complementing pairs of these yeast ALAS mutants for DNA sequencing, and the arrangement of the mutations in each complementation pair revealed that mutants with mutation(s) close to the N-terminus of the enzyme can complement mutants with mutation(s) close to the C-terminus (Figure 5). The results can be interpreted by the intersubunit active site arrangement, in which an active site consists of catalytically essential residues from two different subunits. In addition, glycine 344 and threonine 452 in yeast ALAS are conserved among all known ALAS sequences, and mutations G344C and T452R abolished yeast ALAS activity. These findings suggest that glycine 344 and threonine 452 may play important structural and/or functional role(s) in ALAS.

The sequence for the mouse erythroid ALAS exhibits a high degree of homology with all other known sequences for ALAS, ranging from bacteria to man. Although no significant primary structure identity has been found between ALAS and other PLP-dependent enzymes, recent profile analyses of all known PLP-dependent enzymes led to the inclusion of ALAS in the α subfamily, to which belong aspartate aminotransferase and enzymes that catalyze primarily C2 stereoselectivity reactions (Metha et al., 1993; Alexander et al., 1994; John, 1995). Grishin et al. (1995), using similarities in sequence, secondary structure, hydrophobicity profiles, and structural similarities with enzymes of known structure, classified all of the sequenced PLP-dependent enzymes into seven fold types. Significantly, out of the 312 reported PLP-dependent enzyme sequences, only nine three-dimensional structures are available (Grishin et al., 1995), and seven out of the nine known three-dimensional structures belong to fold I, which includes aspartate aminotransferase and ALAS. Sequence alignments between ALAS and three members of the α family (i.e., glycine hydroxymethyltransferase, glycine *C*-acetyltransferase and 8-amino-7-oxononaanoate synthase) indicate that the mouse erythroid ALAS arginine 149 and lysine 313 residues are invariant in the other three enzymes. Thus, similar contributions by amino acid residues of the two subunits and similar active site arrangements are likely to be shared among the α family of PLP-dependent enzymes.

In summary, by utilizing *in vivo* complementation approaches in which two catalytically inactive ALAS mutants are coexpressed in *E. coli* and combining the data with DNA sequencing results from yeast ALAS mutants, we provide conclusive evidence that ALAS has an intersubunit arrangement with its two active sites located at the interface between subunits and that the active site consists of catalytically essential residues from different subunits.

ACKNOWLEDGMENT

We thank Dr. R. Labbe-Bois for the generous gifts of the yeast ALAS wild-type and mutant genomic DNA, J. Gong for providing the original ALAS alignment data and the plasmid pJGR149A, and G. A. Hunter for developing the nickel chelate agarose chromatography method for the purification of histidine-tagged wild-type ALAS.

REFERENCES

Adams, E. (1979) *Methods Enzymol. 62*, 407−410.
Alexander, F. W., Sandmeier, E., Mehta, P. K., & Christen, P. (1994) *Eur. J. Biochem. 219*, 953−960.
Bishop, D. F. (1990) *Nucleic Acids Res. 18*, 7187−7188.
Bishop, D. F., Henderson, A. S., & Astrin, K. H. (1990) *Genomics 7*, 207−214.
Borthwick, I. A., Srivastava, G., Day, A. R., Pirola, B. A., Snoswell, M. A., May, B. K., & Elliott, W. H. (1985) *Eur. J. Biochem. 150*, 481−484.
Bradshaw, R. E., Dixon, S. W., Raitt, D. C., & Pillar, T. M. (1993) *Curr. Genet. 23*, 501−507.
Chang, A. C. Y., & Cohen, S. N. (1978) *J. Bacteriol. 134*, 1141−1156.
Cox, T. C., Bawden, M. J., Abraham, N. G., Bottomley, S. S., May, B. K., Baker, E., Chen, L. Z., & Sutherland, G. R. (1990) *Am. J. Hum. Genet. 46*, 107−111.
Cox, T. C., Bawden, M. J., Martin, A., & May, B. K. (1991) *EMBO J. 10*, 1891−1902.
Drolet, M., & Sasarman, A. (1991) *Mol. Gen. Genet. 226*, 250−256.
Ferreira, G. C., & Dailey, H. A. (1993) *J. Biol. Chem. 268*, 584−590.

Ferreira, G. C., & Gong, J. (1995) *J. Bioenerg. Biomembr. 27*, 151−159.
Ferreira, G. C., Neame, P. J., & Dailey, H. A. (1993) *Protein Sci. 2*, 1959−1965.
Gong, J., & Ferreira, G. C. (1995) *Biochemistry 34*, 1678−1685.
Grishin, N. V., Phillips, M. A., & Goldsmith, E. J. (1995) *Protein Sci. 4*, 1291−1304.
Hunter, G. A., & Ferreira, G. C. (1995) *Anal. Biochem. 226*, 221−224.
John, R. A. (1995) *Biochim. Biophys. Acta 1248*, 81−96.
Jordan, P. M. (1991) in *Biosynthesis of Tetrapyrroles* (Jordan, P. M., Ed.) pp 1−66, Elsevier, Amsterdam.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Li, J.-M., Brathwaite, O., Cosloy, S., & Russell, C. S. (1989) *J. Bacteriol. 171*, 2547−2552.
Metha, P. S., Hale, T. I., & Christen, P. (1993) *Eur. J. Biochem. 214*, 549−561.
McClung, C. R., Somerville, J. E., Guerinot, M. L., & Chelm, B. K. (1987) *Gene 54*, 133−139.
Munakata, H., Yamagami, T., Nagai, T., Yamamoto, M., & Hayashi, N. (1993) *J. Biochem. (Tokyo) 114*, 103−111.
Neidle, E. L., & Kaplan, S. (1993) *J. Bacteriol. 175*, 2292−2303.
Page, M. D., & Ferguson, S. J. (1994) *J. Bacteriol. 176*, 5919−5928.
Riddle, R. D., Yamamoto, M., & Engel, J. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 792−796.
Rose, R. E. (1988) *Nucleic Acids Res. 16*, 355.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Sambrook, J., Frisch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY.
Schoenhaut, D. S., & Curtis, P. J. (1986) *Gene 48*, 55−63.
Schoenhaut, D. S., & Curtis, P. J. (1989) *Nucleic Acids Res. 17*, 7013−7028.
Sutherlabd, G. R., Baker, E., Callen, D. F., Hyland, V. J., May, B. K., Healy, H. M., & Borthwick, I. A. (1988) *Am. J. Hum. Genet. 43*, 331−335.
Urban-Grimal, D., & Labbe-Bois, R. (1981) *Mol. Gen. Genet. 183*, 85−92.
Urban-Grimal, D., Volland, C., Garnier, T., Dehoux, P., & Labbe-Bois, R. (1986) *Eur. J. Biochem. 156*, 511−519.
Wright, M. S., Eckert, J. J., Biel, S. W., & Biel, A. J. (1991) *FEMS Microbiol. Lett. 78*, 339−342.
Yamamoto M., Kure, S., Engel, J. D., & Hiraga, K. (1988) *J. Biol. Chem. 263*, 15973−15979.

BI952918M