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The role of tyrosine 121 in cofactor binding of 5-aminolevulinate synthase

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Abstract

5-Aminolevulinate synthase (EC 2.3.1.37) is the first enzyme in the heme biosynthesis in nonplant eukaryotes and some prokaryotes. It functions as a homodimer and requires pyridoxal 5′-phosphate as an essential cofactor. Tyr-121 is a conserved residue in all known sequences of 5-aminolevulinate synthases. Further, it corresponds to Tyr-70 of *Escherichia coli* aspartate aminotransferase, which has been shown to interact with the cofactor and prevent the dissociation of the cofactor from the enzyme. To test whether Tyr-121 is involved in cofactor binding in murine erythroid 5-aminolevulinate synthase, Tyr-121 of murine erythroid 5-aminolevulinate synthase was substituted by Phe and His using site-directed mutagenesis. The Y121F mutant retained 36% of the wild-type activity and the K_m value for substrate glycine increased 34-fold, while the activity of the Y121H mutant decreased to 5% of the wild-type activity and the K_m value for glycine increased fivefold. The pK_{a1} values in the pH-activity profiles of the wild-type and mutant enzymes were 6.41, 6.54, and 6.65 for wild-type, Y121F, and Y121H, respectively. The UV-visible and CD spectra of Y121F and Y121H mutants were similar to those of the wild-type with the exception of an absorption maximum shift (420 → 395 nm) for the Y121F mutant in the visible spectrum region, suggesting that the cofactor binds the Y121F mutant enzyme in a more unrestrained manner. Y121F and Y121H mutant enzymes also exhibited lower affinity than the wild-type for the cofactor, reflected in the K_d values for pyridoxal 5′-phosphate (26.5, 6.75, and 1.78 μM for Y121F, Y121H, and the wild-type, respectively). Further, Y121F and Y121H proved less thermostable than the wild type. Taken together, these findings indicate that Tyr-121 plays a critical role in cofactor binding of murine erythroid 5-aminolevulinate synthase.

Keywords: ALAS, cofactor binding; PLP; site-directed mutagenesis; Tyr-121

5-Aminolevulinate synthase (ALAS, 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). ALAS functions as a homodimer with two active sites residing at the subunit interface and containing catalytically important residues from two subunits (Tan & Ferreira, 1996). ALAS requires pyridoxal 5′ phosphate (PLP) as an essential cofactor.

ALAS is a member of the α subfamily of PLP-dependent enzymes, which includes aspartate aminotransferase (AAT) and other enzymes catalyzing the primarily Cα stereoselectivity reactions (Alexander et al., 1994). In addition, Grishin et al. (1995) classified all PLP-dependent enzymes with known sequences into sevenfold types (i.e., fold I–VII) and placed ALAS in the same fold as that of AAT (i.e., fold I). The X-ray crystal structure of *Escherichia coli* AAT revealed a conserved tyrosine residue (Y70) involved in cofactor PLP binding, which corresponds to the invariant Tyr-121 residue in murine erythroid ALAS (Kirsch et al., 1984; Smith et al., 1989).

Tyr-70 in AAT is positioned close to the 5′-phosphate group of PLP, and the Tyr-70 hydroxyl group donates a hydrogen bond to the phosphate oxygen OP2 of PLP, which is one of the specific interactions responsible for anchoring the cofactor to the enzyme (Smith et al., 1989). Tyr-70 in *E. coli* AAT has been replaced with Phe using site-directed mutagenesis (Toney & Kirsch, 1987, 1991a, 1991b). The Y70F mutant retained 8% k_{cat} of the wild-type AAT value for the transamination of aspartate and α-ketoglutarate. The

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Abbreviations: ALA, 5-aminolevulinate; ALAS, 5-aminolevulinate synthase; PMP, pyridoxamine 5′-phosphate; PLP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

	121												
Mouse erythroid	V	W	C	S	N	D	**Y**	L	G	I	S	R	H
Human housekeeping	V	W	C	S	N	D	**Y**	L	G	M	S	R	H
Human erythroid	V	W	C	S	N	D	**Y**	L	G	M	S	R	H
Rat housekeeping	V	W	C	S	N	D	**Y**	L	G	M	S	R	H
Rat erythroid	V	W	C	S	N	D	**Y**	L	G	I	S	R	H
Chicken housekeeping	V	W	C	S	N	D	**Y**	L	G	M	S	R	H
Chicken erythroid	L	W	C	S	S	D	**Y**	L	G	L	S	R	H
Saccharomyces cerevisiae hem1	V	W	C	S	N	D	**Y**	L	A	L	S	K	H
Aspergillus nidulans	V	W	C	S	N	D	**Y**	L	G	M	G	R	N
Paracocus denitrificans	V	W	C	G	N	D	**Y**	L	G	M	G	Q	H
Rhodobacter spheroides hemT	V	W	C	S	N	D	**Y**	L	G	M	G	Q	N
Rhodobacter spheroides hemA	V	W	C	G	N	D	**Y**	L	G	M	G	Q	H
Rhodobacter capsulatus	V	W	C	G	N	D	**Y**	L	G	M	G	Q	H
Agrobacterium radiobacter	V	W	C	S	N	D	**Y**	L	G	M	G	Q	N
Bradyrhizobium japonicum	I	W	C	S	N	D	**Y**	L	G	M	G	Q	H

Fig. 1. Alignment of all known ALAS sequences around murine erythroid ALAS Tyr-121 residue. Nonidentical amino acids are underlined (adapted from Ferreira & Gong, 1995).

affinity for cofactor pyridoxamine 5′-phosphate (PMP) and PLP decreased 27- and 100-fold, respectively, compared to the wild type. In addition, Tyr-70 of chicken mitochondrial AAT has also been substituted by His using site-directed mutagenesis. The activity of Y70H decreased to 13% of the wild type. However, the dissociation constants of PMP and PLP for the mutant enzyme increased only 2.6- and 1.3-fold, respectively, compared to the wild type, suggesting that in chicken mitochondrial AAT, His-70 to some extent can replace Tyr-70 in forming a hydrogen bond to the cofactor.

Tyr-121 of murine erythroid ALAS is invariant in all known ALAS sequences and corresponds to the conserved Tyr-70 residue in AAT. To investigate whether Tyr-121 in murine erythroid ALAS plays a similar role in cofactor binding to that of AAT Tyr-70, we replaced the Tyr residue with Phe and His using site-directed mutagenesis, and we have studied the catalytic and physical properties of the resultant Y121F and Y121H ALAS mutant enzymes. The results indicate that Tyr-121 plays an important role in cofactor PLP binding in murine erythroid ALAS.

Results

Alignment of all known ALAS sequences around murine erythroid ALAS Tyr-121

Protein sequence alignment demonstrated that murine erythroid ALAS Tyr-121 is conserved in all known ALASs (Fig. 1, adapted from Ferreira & Gong, 1995). Further, sequence comparison of the murine erythroid ALAS with the *E. coli* AAT indicated that Tyr-121 might correspond to the AAT Tyr-70, which has been shown to be an invariant residue in all AATs and to be involved in cofactor binding (Toney & Kirsch, 1987, 1991a, 1991b; Pan et al., 1994). Tyr-121 was, therefore, substituted by Phe and His using site-directed mutagenesis to investigate whether this Tyr residue is important to the cofactor PLP binding in ALAS.

Kinetic studies of ALAS Y121F and Y121H mutants

Kinetic parameters were determined for the purified Y121F and Y121H mutants (Table 1). The Y121F mutant retained 36% of the wild-type activity, and the K_m value for substrate glycine increased 34-fold compared to the wild type. The activity of the Y121H mutant decreased to 5% activity of the wild type, while the K_m value for glycine increased only fivefold. Kinetic parameters were also measured at different pH values (pH 6.5–9.5). The apparent K_ms for glycine and succinyl CoA are shown in Figure 2, and demonstrate that higher pH values cause stronger binding for both substrates by ALAS. The pH dependencies of k_{cat} for the wild-type, Y121F, and Y121H are illustrated in Figure 3. The data were fitted to the bell-shaped curves described by Equation 3. The calculated pK_{a1}s are 6.41, 6.54, and 6.65 for the wild type, Y121F, and Y121H, respectively; the calculated pK_{a2}s are 8.32, 8.15, and 8.16 for the wild type, Y121F, and Y121H, respectively.

UV-visible and CD spectroscopic properties

The absorption spectra of Y121 mutants and the wild type exhibited similar absorption maxima at 277 and 330 nm (Fig. 4A,B). Y121H mutant and the wild type also had another absorption maximum at 410 nm, while this absorption maximum shifted to 395 nm in Y121F mutant. The wild-type and mutant apoenzymes displayed absorption maxima only at 278 nm (Fig. 4C). Absorption maxima at 330 and 410 nm correspond to different ionization states of the internal aldimine bond between the PLP and the ALAS (either wild type or mutants). The two absorption maxima, at 330 and 410 nm, represent the unprotonated and protonated forms, respectively. CD spectra of the Y121 mutant and wild-type holoenzymes in the visible region are illustrated in Figure 5A. The Y121 mutants exhibited CD spectra similar to that of the wild-type enzyme. They all displayed a strong positive CD maximum around 425 nm, which reflects the asymmetric orientation of the bound PLP cofactor. Similar CD spectra in the UV region (200–270 nm) were also recorded for the mutant and wild-type holoenzymes (data not shown), demonstrating that the mutations did not induce substantial changes in the enzyme conformation. No CD maxima were detected in the visible region for the ALAS mutant and wild-type apoenzymes (Fig. 5). CD spectra in the UV region were also recorded for the apoenzymes, and all apoenzymes exhibited sim-

Table 1. *Kinetic parameters for Y121 mutants of ALAS*

	K_m^{Gly} (mM)	$K_m^{S.CoA}$ (μM)	V_{max} (nmol·mg^{-1}·h^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m^{Gly} (mM^{-1}·min^{-1})	$k_{cat}/K_m^{S.CoA}$ (μM^{-1}·min^{-1})
Wild type	11.7	2.03	4.23×10^4	39.5	3.38	19.5
Y121F	400	14.0	1.51×10^4	14.1	0.0353	1.01
Y121H	52.2	1.60	2.28×10^3	2.13	0.0408	1.33

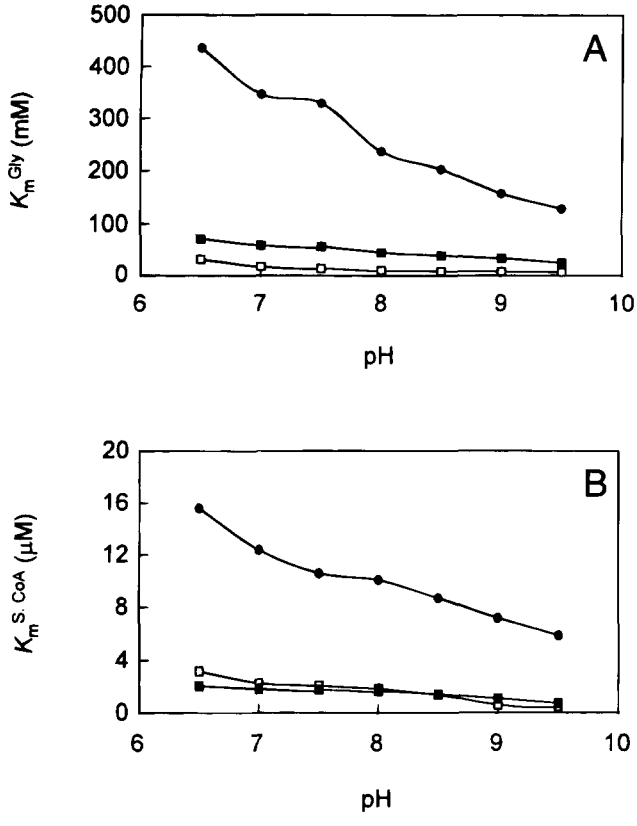

Fig. 2. pH dependence of substrate binding for ALAS Y121 mutants. Apparent K_m values of glycine (**A**) and succinyl CoA (**B**) for wild-type, Y121F and Y121H mutants were measured at pH 6.5–9.5. The pH was adjusted by adding acetic acid or NaOH into the buffer containing 20 mM imidazole and 20 mM AMPSO. Symbols: open squares, wild type; filled circles, Y121F; filled squares, Y121H.

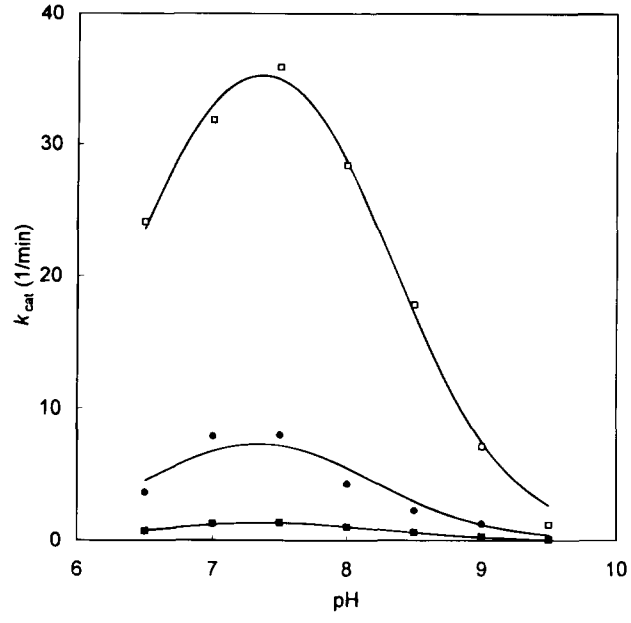

Fig. 3. pH dependence of k_{cat} for ALAS Y121 mutants. Apparent k_{cat} values for wild-type, Y121F and Y121H mutants were measured at pH 6.5–9.5. The pH was adjusted by adding acetic acid or NaOH into the buffer containing 20 mM imidazole and 20 mM AMPSO. Symbols: open squares, wild type; filled circles, Y121F; filled squares, Y121H.

ilar CD spectra in the UV region to those of holoenzymes (data not shown), suggesting that the removal of the PLP cofactor had little effect on the overall structure of ALAS.

PLP cofactor affinity of Y121 mutants

Titration of ALAS wild-type and mutant apoenzymes resulted in positive changes in the apparent ellipticities at 425 nm of the CD spectra, which were plotted against PLP concentrations for each ALAS sample. K_d^{PLP} values were determined for the wild-type and mutant enzymes by a theoretical curve fit of the experimental data using Equation 2 (Fig. 6). The obtained K_d^{PLP} values were 1.78, 26.5, and 6.75 μM for the wild type, Y121F, and Y121H, respectively.

Thermostability characterization of Y121 mutants

Thermostability experiments were conducted to determine the relative thermostability of Y121 mutant enzymes. The enzymatic activity was plotted as a function of temperature, which is shown in Figure 7. The calculated thermotransition temperature $T_{1/2}$, which is defined as the temperature needed to achieve a 50% activity loss, was 55.3, 53.2, and 54.6 °C for the wild type, Y121F, and Y121H, respectively.

Discussion

Both ALAS and AAT belong to the same predicted fold type group (i.e., fold type I) and the α subfamily of the PLP-dependent enzymes (Alexander et al., 1994, Grishin et al., 1995). The availability of AAT high-resolution crystal structures and the knowledge of functions of many of its active site residues enable sequence alignment with AAT to be an efficient tool to predict the function(s) of a specific amino acid residue in ALAS. Protein sequence alignment permitted us to propose that Tyr-121 of murine erythroid ALAS forms a hydrogen bond with the phosphate group of cofactor PLP. Tyr-121, therefore, was mutated to Phe and His. The activity of Y121F mutant retained 36% of that of the wild type. K_m for succinyl CoA increased sevenfold, while K_m for glycine increased 34-fold. The activity of Y121H mutant decreased to 5% that of the wild type. K_m for succinyl CoA did not change significantly, while the K_m for glycine increased fivefold. Apparently, the Y121H mutant exhibits much greater substrate binding capability compared to the Y121F mutant. One possible reason for the high K_m^{Gly} value in ALAS Y121F mutant is that the Tyr → Phe mutation eliminates the hydrogen bond between the Tyr-121 hydroxyl group and cofactor PLP, which weakens the binding of PLP to ALAS and in turn affects the binding of glycine to ALAS, because glycine forms an external aldimine with PLP. To some extent His-121 may replace Tyr-121 in forming a hydrogen bond with PLP, which exhibits a positive effect on the binding of glycine to ALAS. In addition, the pH dependencies of the kinetic parameters of the Y121 mutant and wild-type enzymes were also investigated at pH 6.5–9.5. Higher pH resulted in better binding of both substrates for the mutants and wild type (Fig. 2A,B). The improved binding affinity of glycine may result from a combination of the increased amount of the deprotonated form of glycine and the decreased amount of the Schiff base protonated form when the pH is changed

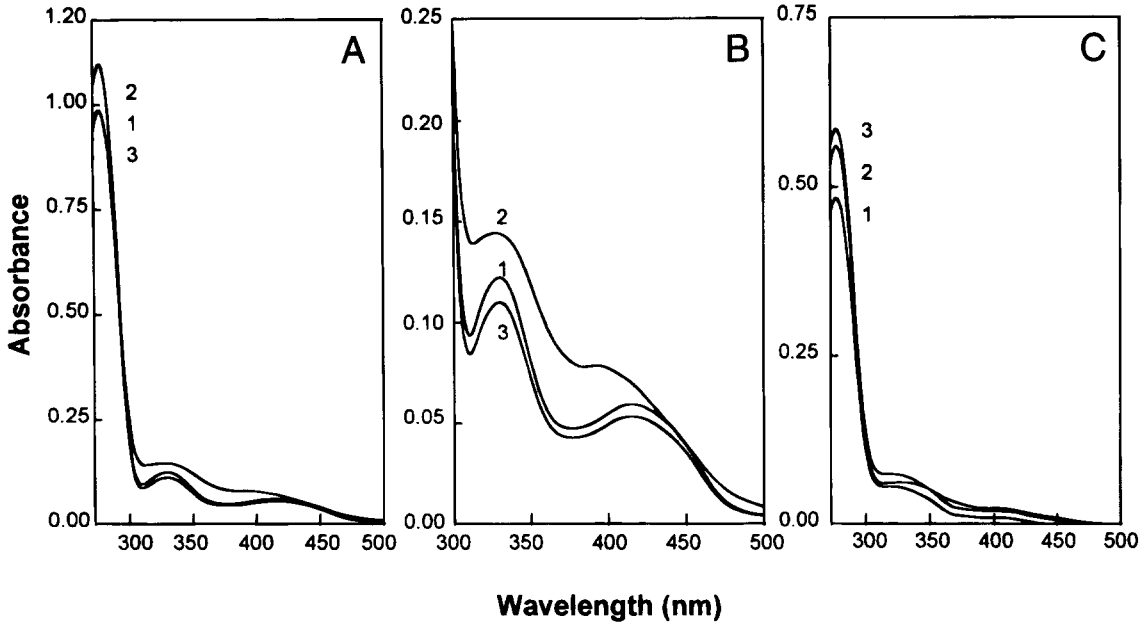

Fig. 4. UV-visible absorption spectra of ALAS Y121 mutants. UV-visible absorption spectra were recorded in 20-mM imidazole buffer, pH 7.2. **A:** ALAS holoenzyme of the wild-type and mutant forms. Curves: 1, wild type (15.1 μM); 2, Y121F (17.8 μM); 3, Y121H (16.4 μM). **B:** Absorption spectra of PLP bound to ALAS (enlargement of the visible region 300–550 nm in **A**). **C:** absorption spectra of ALAS apoenzymes. Concentrations: wild type (7.5 μM); Y121F (10.2 μM); Y121H (11.5 μM). Number labels in **B** and **C** are as in **A**.

from a low to a high value. As with the wild-type enzyme, maximum activity was attained between 7.0 and 8.0 for the Y121 mutants. The pH-rate profiles of the mutant and wild-type enzymes can be fitted approximately to a bell-shaped curve with pK_{a1} 6.41, 6.54, and 6.65 for the wild type, Y121F, and Y121H, respectively (Fig. 3). In chicken mitochondrial AAT, when the pH was lowered to 5.5, the activity of the Y70H mutant was abolished, while the wild type still retained 75% of the maximum activity (Pan et al., 1994). The pK_{a1} value of Y70H was determined to be 6.3, probably due to the newly introduced His residue. In ALAS, we could only lower the pH to 6.5, because the enzyme (either wild type or mutants) started to precipitate below pH 6.5. Nevertheless, there is a 0.24 pH difference between the pK_{a1} values of the wild type (6.41) and the Y121H mutant (6.65), probably also attributed to the newly introduced His residue. The pK_{a2} values are 8.32, 8.15, and 8.16 for the wild type, Y121F, and Y121H, respectively, probably reflecting the deprotonation of the active-site Lys-313 residue that forms the Schiff base linkage with cofactor PLP.

The UV-visible absorption and CD spectra were similar for the Y121 mutants and the wild-type enzyme with the exception of the Y121F mutant in the absorption region of 350–450 nm. A shift of absorption maximum from 420–395 nm was observed for the Y121F mutant, suggesting that the PLP cofactor in the protonated form of the internal aldimine exists more like a free PLP molecule in the Y121F mutant.

The Y121F mutation resulted in a 15-fold decrease in the affinity of cofactor PLP for ALAS (Fig. 6), demonstrating that the proposed hydrogen bond between the hydroxyl group of Tyr-121 and the phosphate group of PLP indeed contributes significantly to the cofactor binding. In contrast, the Y121H mutation only caused a fourfold decrease in the affinity of cofactor PLP (Fig. 6), suggesting that a hydrogen bond might be to some extent rebuilt between His-121 and the cofactor. Finally, the Y121 mutants exhibited lower thermostability than the wild type.

In summary, using site-directed mutagenesis, kinetic studies and cofactor PLP titration, we provide conclusive evidence that Tyr-121 in murine erythroid ALAS plays an important role in cofactor binding, similar to that of Tyr-70 in *E. coli* AAT.

Materials and methods

Materials

Restriction enzyme *EcoR*I was obtained from New England Biolabs (Beverly, MA) and was used according to the supplier's instructions. Oligonucleotide primers were synthesized by Cybersyn (Lenni, PA). Chameleon mutagenesis kit was a product from Stratagene (La Jolla, CA). Sequenase and sequencing kit were from U.S. Biochemical Corp. (Cleveland, OH). [α-^{35}S]-dATP was from Dupont/NEN Research Products (Boston, MA). Acrylamide and gel reagents were purchased from BioRad (Hercules, CA). The bicinchoninic acid protein assay reagents were obtained from Pierce Chemical Co (Rockford, IL). Ni-NTA agarose was purchased from Qiagen (Valencia, CA). All other chemicals were of the highest purity available.

Methods

Mutagenesis, expression, and purification of Y121F and Y121H

Plasmid pDT6, which contains a full-length mouse erythroid ALAS wild-type sequence with a N-terminal extension consisting of five histidine residues (Tan & Ferreira, 1996), was purified from its *E. coli* DH5α host cell. Single-stranded DNA of this plasmid

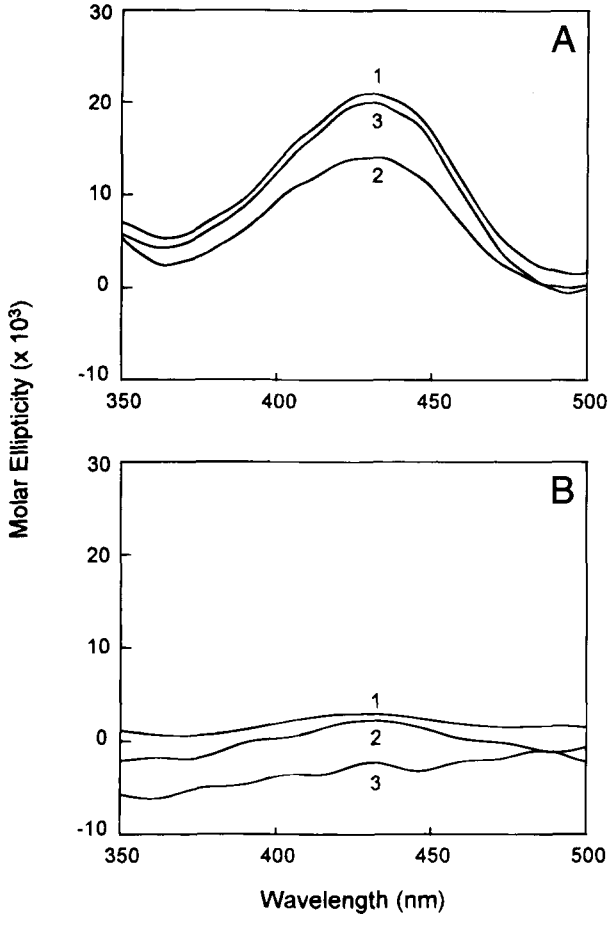

Fig. 5. CD spectra of PLP bound to the active site of ALAS Y121 mutants. Spectra were recorded in 20-mM imidazole buffer, pH 7.2. **A:** Holoenzymes. Curves: 1, wild type (52.7 μM); 2, Y121F (39.0 μM); 3, Y121H (47.0 μM). **B:** Apoenzymes. Concentrations: wild type (7.5 μM); Y121F (10.2 μM); Y121H (11.5 μM). Number labels in **B** are as in **A**.

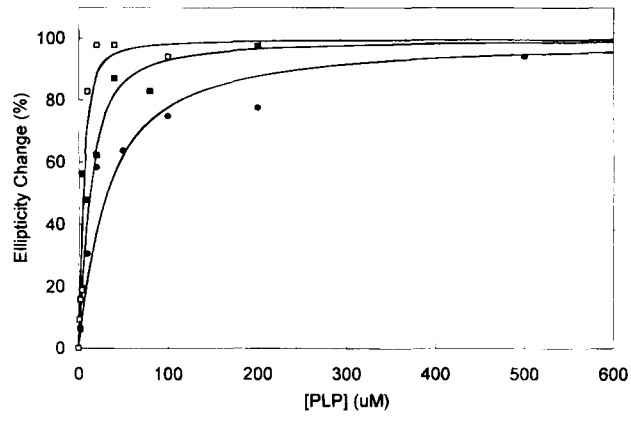

Fig. 6. CD titration of apo wild-type and Y121 mutant enzymes with PLP at pH 7.2, 25 °C. The titration was performed by recording CD spectra of ALAS enzymes in the presence of 0–500 μM PLP. Apoenzymes were prepared in 20 mM imidazole buffer, pH 7.2. Symbols: open squares, wild type; filled circles, Y121F; filled squares, Y121H.

was prepared according to the alkaline lysis method (Sambrook et al., 1989). Site-directed mutagenesis was performed on the single-stranded DNA using the Chameleon mutagenesis kit from Stratagene. The mutagenic oligonucleotides for Y121F and Y121H were 5′-GGT GTA GTA ATG ACT TTT TGG GCA TAA GC-3′ and 5′-GGT GTA GTA ATG ACC ATT TGG GCA TAA GC-3′, respectively. Clones obtained after the mutagenesis procedure were screened using DNA sequencing, as described in the dideoxynucleotide chain termination method (Sanger et al., 1977). The resulting expression plasmids containing the mutation Y121F or Y121H were named pDT10 and pDT14, respectively. The mutant and wild-type enzymes were overproduced in *E. coli* DH5α host strain harboring the different expression plasmids and purified to homogeneity using nickel chelate agarose chromatography (Tan & Ferreira, 1996).

SDS-PAGE, protein concentration determination, kinetic studies, UV-visible, and CD spectra

SDS-PAGE and protein concentration determination were performed as described previously in Tan and Ferreira (1996). Briefly, 15% acrylamide and 1.5-mm-thick gels were used in SDS-PAGE and the protein concentrations were determined by the bicinchoninic acid assay according to the manufacturer's instructions. A continuous spectrophotometric assay was used to measure ALAS enzyme activity (Hunter & Ferreira, 1995) with a modification of temperature to 37 °C. Hanes and secondary plots were employed to obtain k_{cat} and K_m values, which were determined for glycine and succinyl-CoA at constant concentrations of 1–72 μM for succinyl-CoA and 10–1,000 mM for glycine, respectively. A Shimadzu UV2100U spectrophotometer was used to measure the UV-visible absorption spectra of the wild-type and mutant ALASs. UV and visible CD spectra for the wild-type and mutant ALAS enzymes were obtained using a Jasco J710 spectropolarimeter, as previously described by Gong et al. (1996).

Preparation of Apo ALAS enzymes and determination of dissociation constants (K_d) of PLP

Wild-type and mutant ALAS apoenzymes were prepared using extensive dialysis (Gong et al., 1996). Protein samples were di-

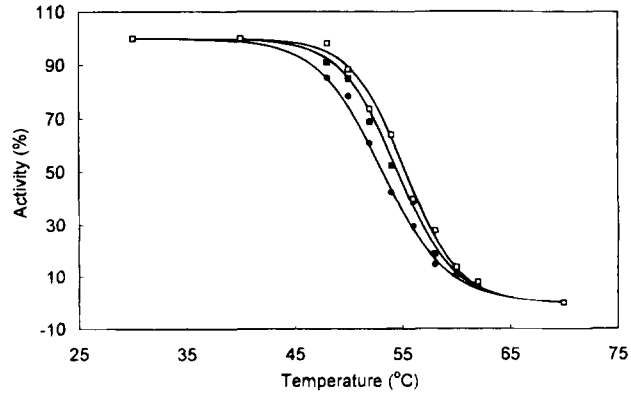

Fig. 7. Thermostability of ALAS wild-type and Y121 mutant enzymes. ALAS samples were incubated at a given temperature for 3 min, then cooled to 0 °C, and activity assays performed. The enzymatic activity from the sample treated at 0 °C was set as 100%. Symbols: open squares, wild type; filled circles, Y121F; filled squares, Y121H.

luted to around 4 μM and the dialysis was carried out at 4 °C. Ten milliliters of ALAS samples were dialyzed against 500 mL 20 mM imidazole buffer without PLP, with multiple changes of the buffer throughout the dialysis. After extensive dialysis, activity assay and UV-visible spectrum analyses were conducted to evaluate the dialysis result. The apo ALAS samples were concentrated using Amicon ultrafiltration cells with a YM10 membrane. The K_d^{PLP} values were determined by CD spectrometric titration (in the visible region) of ALAS apoenzymes with PLP (Gong et al., 1996). Basically, ALAS wild-type and mutant apoenzymes were taken in 20 mM imidazole buffer containing 20% glycerol at a concentration range of 7–11 μM. A series of high concentrations of PLP were used in the experiments to ensure less than 5% dilution of the protein samples. CD spectra were recorded from 400–550 nm after each addition of PLP. The binding of PLP was monitored by a CD spectrum increase at 425 nm and the increases were used to calculate the K_d^{PLP} values. K_d for PLP is defined as

$$K_d = ([ALAS]_{apo}[PLP]_{ub})/[ALAS]_{holo} \quad (1)$$

where $[PLP]_{ub}$ is the concentration of the unbound PLP cofactor, and $[ALAS]_{apo}$ and $[ALAS]_{holo}$ represent the concentrations of the apo and holo ALAS, respectively. The ratio R of holo ALAS to total ALAS is derived as

$$R = \left(\frac{1}{2}\right)\left\{1 + \frac{K_d}{[ALAS]_t} + \frac{[PLP]_t}{[ALAS]_t} - \sqrt{\left(1 + \frac{K_d}{[ALAS]_t} + \frac{[PLP]_t}{[ALAS]_t}\right)^2 - \frac{4[PLP]_t}{[ALAS]_t}}\right\} \quad (2)$$

where $[ALAS]_t$ is the total ALAS concentration and $[PLP]_t$ is the total PLP concentration. Changes in the apparent ellipticities at 425 nm were plotted against $[PLP]_t$, and K_d was calculated by a curve fit of the experimental data using Equation 2.

Determination of the thermostability of ALAS Y121 mutants

ALAS samples with 5 μM protein concentration were incubated at a given temperature for 3 min, then cooled to 0 °C, using a MJ Research MiniCycler. Activity assays were conducted on the treated samples at 37 °C with 24 μM succinyl-CoA and 300 mM glycine for the wild-type and Y121H mutant, and 72 μM succinyl-CoA and 1 M glycine for the Y121F mutant. The enzymatic activity from the sample treated at 0 °C was normalized as 100%. The thermotransition temperature, $T_{1/2}$, is defined as the temperature needed to achieve 50% activity.

Evaluation of the pH dependence of the kinetic parameters

Data for the pH dependence of k_{cat} were fitted to the bell-shaped curve described by

$$k_{cat} = \frac{k_{cat\,OPT}}{1 + 10^{(pK_{a1} - pH)} + 10^{(pH - pK_{a2})}} \quad (3)$$

where $k_{cat\,OPT}$ is k_{cat} at the optimal pH, and K_{a1} is the ionization constant of the enzyme complex that affects the acid side of the pH curve, and K_{a2} is that which affects the alkaline side (Gloss & Kirsch, 1995).

Acknowledgments

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