Effects of the E177K Mutation in D-Amino Acid Transaminase. Studies on an Essential Coenzyme Anchoring Group That Contributes to Stereochemical Fidelity[†,‡]

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ABSTRACT: D-Amino acid transaminase is a bacterial enzyme that uses pyridoxal phosphate (PLP) as a cofactor to catalyze the conversion of D-amino acids into their corresponding α-keto acids. This enzyme has already been established as a target for novel antibacterial agents through suicide inactivation by a number of compounds. To improve their potency and specificity, the detailed enzyme mechanism, especially the role of its PLP cofactor, is under investigation. Many PLP-dependent transaminases have a negatively charged amino acid residue forming a salt-bridge with the pyridine nitrogen of its cofactor that promotes its protonation to stabilize the formation of a ketimine intermediate, which is subsequently hydrolyzed in the normal transaminase reaction pathway. However, alanine racemase has a positively charged arginine held rigidly in place by an extensive hydrogen bond network that may destabilize the ketimine intermediate, and make it too short-lived for a transaminase type of hydrolysis to occur. To test this hypothesis, we changed Glu-177 into a titratable, positively charged lysine (E177K). The crystal structure of this mutant shows that the positive charge of the newly introduced lysine side chain points away from the nitrogen of the cofactor, which may be due to electrostatic repulsions not being overcome by a hydrogen bond network such as found in alanine racemase. This mutation makes the active site more accessible, as exemplified by both biochemical and crystallographic data: CD measurements indicated a change in the microenvironment of the protein, some SH groups become more easily titratable, and at pH 9.0 the PMP peak appeared around 315 nm rather than at 330 nm. The ability of this mutant to convert L-alanine into D-alanine increased about 10-fold compared to wild-type and to about the same extent as found with other active site mutants. On the other hand, the specific activity of the E177K mutant decreased more than 1000-fold compared to wild-type. Furthermore, titration with L-alanine resulted in the appearance of an enzyme−substrate quinonoid intermediate absorbing around 500 nm, which is not observed with usual substrates or with the wild-type enzyme in the presence of L-alanine. The results overall indicate the importance of charged amino acid side chains relative to the coenzyme to maintain high catalytic efficiency.

The recently solved structure of bacterial D-amino acid transaminase reveals important interactions of the coenzyme, pyridoxal 5′-phosphate (PLP),[1] with protein side chains that provide this enzyme with the ability to catalyze reactions with D- rather than with L-amino acids (*1*). However, the details of this stereochemical fidelity remain to be elucidated. As part of an effort to understand this process, we have been systematically replacing these anchoring amino acids to test the catalytic and stereochemical properties of the recombinant enzymes. Wild-type enzyme showed a high preference for D-alanine rather than L-alanine (*2*), which was reduced to a large extent when the PLP-binding residue Lys-145 was replaced by Gln (*3, 4*). Replacement of Tyr-31, a residue involved in the interaction with the phenolic oxygen of PLP, with a Gln led to a reduced stereochemical fidelity and catalytic efficiency (*5*). However, the replacement of Ser-180, which is thought to be part of the side chain trapping pocket, with Ala had very little effect on stereochemical preference or activity (*5*).

One of the significant differences in the structures of the active sites of D-amino acid transaminase and alanine racemase is the substitution of a negatively charged Glu in the former enzyme for a positively charged Arg in the latter near the pyridinium nitrogen of the PLP ring (*6*). Accordingly, we have substituted Glu-177 of D-amino acid transaminase with a Lys (E177K) in order to have a side chain in the titratable range of pH and to study the effects on catalytic efficiency, stereochemical fidelity, circular dichro-

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[‡] Atomic coordinates are available from the Brookhaven Protein Data Bank under entry code 5DAA.

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine phosphate; TFA, trifluoroacetic acid; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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ism properties, SH titration, and X-ray crystallographic structure.

EXPERIMENTAL PROCEDURES

DNA, Phage, Bacterial Strains, Enzymes, and Site-Directed Mutagenesis. These experimental procedures have been described previously (*7*, *8*). The oligonucleotide was synthesized at the Rockefeller University Protein Sequence Facility, where the DNA sequence was also verified. The mismatched base located approximately in the center of the sequence is given in italics. The following oligonucleotide was used:

5′-TGA-AGA-AGA-GCC-TT*T*-TGT-TAC-TGT-ATT-3′

The procedures for the preparation of the plasmids for the site-directed mutagenesis experiments and for the expression of the enzymes have also been described previously (*7*, *8*).

Purification of the Enzyme. Both wild-type as well as mutant enzymes were purified by slight modifications of the procedures as described by van Ophem et al. (*5*). During the heat treatment step of the purification of mutant enzymes, the temperature was lowered to 48 °C rather than the 53 °C used for the wild-type enzyme. The second anion exchange step was performed on a Mono-Q 10/10 column (Pharmacia), using a linear gradient from 0 to 0.3 M KCl in 10 mM potassium phosphate, pH 7.6, supplemented with 50 μM PLP and 0.01% β-mercaptoethanol, at a flow rate of 2 mL/min over a period of 90 min. The enzymes were approximately 99% pure after this step, but, if needed as judged from SDS−PAGE gels, a final gel filtration step was used to remove the last impurities. This step was performed on a Superose-12 column (Pharmacia) equilibrated with 0.1 M potassium phosphate buffer, pH 7.3, containing 50 μM PLP and 0.01% β-mercaptoethanol, at an elution rate of 0.4 mL/min. The purified enzymes gave one major band by native and SDS−PAGE. To remove excess PLP, preparations were dialyzed extensively prior to usage at 4−6 °C against 50 mM potassium phosphate buffer, pH 7.0, or against 50 mM bis-Tris/HCl, pH 7.0, depending on the buffer used in the experiment.

Proteolytic Digests. To verify that the mutant enzyme contained the desired amino acid substitution, 1 mg of the enzyme was carboxymethylated and reduced, as described (*9*). After dialysis against 0.1 M NH_4HCO_3 for 16 h, the protein was digested with 2% TPCK-treated trypsin (Sigma type XIII from bovine pancreas). After lyophilization, the enzyme was dissolved in 0.1% trifluoroacetic acid (TFA), and the peptides were separated on a Vydac Protein and Peptide C-18 column (15 cm × 4.6 mm) by applying a linear gradient from 0.1% TFA to 0.1% TFA/80% acetonitrile at a flow rate of 1 mL/min. The eluent was monitored at 215 nm. Amino acid analysis of the peptide fragment containing the mutation was performed after hydrolysis in 6 N HCl for 20 h at 120 °C and subsequent analysis on a Beckman 6300 Amino Acid Analyzer.

Crystallization. The enzyme was concentrated to 30 mg/mL in 50 mM potassium phosphate buffer, pH 7.3, containing 0.13 M KCl, 0.2 mM EDTA, 50 μM PLP, and 0.01% β-mercaptoethanol. The protein was crystallized by the hanging-drop method with 26% poly(ethylene glycol) 3350 (Sigma Chemical Co., St. Louis, MO), 0.3 M sodium acetate, and 0.1 M Tris/HCl, pH 8.5. Two microliters of protein solution was mixed with 2 μL of the above crystallization solution and suspended at room temperature over 0.5 mL of crystallization solution. Within 2 days, yellow crystals (due to the presence of an internal aldimine) appeared and were allowed to grow for another 3 days. One crystal had dimensions 0.5 × 0.4 × 0.2 mm and was used for data collection. The crystal had the symmetry of the orthorhombic space group $P2_12_12_1$. Its unit cell parameters are $a = 77.9$ Å, $b = 91.9$ Å, and $c = 89.4$ Å, corresponding to a unit cell volume of 640 000 Å^3. The calculated volume per unit mass (V_m) of 2.4 Å^3/Da is also in the expected range.

Table 1: Data for Solution of the Crystal Structure

Crystal Data	
protein	E177K
space group	$P2_12_12_1$
unit cell parameters	
a (Å)	77.9
b (Å)	91.9
c (Å)	89.4
Data Collection	
reflections, observed	38415
relections, unique	13649
R_{sym} (% on I)	14.4
resolution (Å)	100−2.9
I/σ cutoff	>0.0
completeness, overall (%)	93.1
highest resolution shell (Å)	3.2−2.9
completeness, highest resolution shell (%)	94.1
R_{sym} (% on I), highest resolution shell	33.7
Refinement	
resolution (Å)	50−2.9
I/σ cutoff	>0.1
reflections	13752
R_{factor}	18.1
R_{free}	26.0
protein atoms	4466
cofactor atoms	30
water molecules	3
B-factor model	individual
restraints (rms observed)	
bond length (Å)	0.007
bond angles (deg)	1.3
improper angles (deg)	1.2
dihedral angles (deg)	24.0

Solution of the Crystal Structure. Data were collected at 4 °C with a scan width of 1° per frame and an exposure time of 20 min per frame on a RAXIS IIC image plate system mounted on a Rigaku RU-200B X-ray generator running at 45 kV and 140 mA. The data set was collected from a single crystal, and was 99.1% complete to 2.9 Å resolution. Frames were integrated and scaled together using the program XDS (*10*) (Table 1).

The structure was solved by molecular replacement using the native monoclinic structure of the enzyme (*1*) as search probe in the program AMORE, which is part of the CCP4 package (*11*). The program XPLOR (*12*) was then used for all remaining refinement. Due to the low ratio of observables to degrees of freedom, noncrystallographic symmetry was used between the two monomers of the asymmetric unit. Rigid body refinement (of each monomer in the asymmetric unit) was first performed to optimize the initial location. This refinement was followed by several cycles of positional refinement, grouped temperature factor refinement, and placement of water molecules. Water molecules were placed

with PEAKMAX and WATPEAK from the CCP4 package (*13*).

Enzyme Activity. Enzyme activity was measured at room temperature by determining the rate of NADH conversion to NAD at 340 nm in 0.1 M EPPS/KOH, pH 8.1, using the coupled assay with 10 units of lactic dehydrogenase (Sigma) in the presence of 200 mM D-alanine, 25 mM α-ketoglutarate, and 0.1 mM NADH (*14*). Activities were calculated using a molar absorption coefficient of 6200 M^{-1} cm^{-1} for NADH at 340 nm (*15*). One unit of enzyme activity is defined as the conversion of 1 μmol of NADH to NAD/min, which corresponds to the conversion of 1 μmol of pyruvate produced from D-alanine. The specific activity is defined as units per milligram of protein. With L-alanine as substrate, D-alanine was omitted from the activity assay, but all the other components above were present. Protein concentrations were determined by their respective molecular extinction coefficients at 280 nm (wild-type, K145Q, K145N, S180A, and Y31Q: references *8*, *3*, *9*, *5*, and *5*, respectively). Protein concentrations of E177K were determined by using a molar extinction coefficient at 280 nm of 67 200 M^{-1} cm^{-1}, as determined by applying the method as described by van Iersel et al. (*16*). D- or L-glutamate or D- or L-aspartate were tested by incubating 10 mM of these amino acids with 10 mM pyruvate and 0.3 mg of mutant enzyme in 0.1 M EPPS/KOH, pH 8.1, at room temperature for 15 min. Subsequently, samples were taken and mixed with 0.1 volume of ice-cold 50% 5-sulfosalysilic acid, and after 10 min on ice, protein was removed by centrifugation for 10 min at 16000*g*. The pH of the supernatant was adjusted to 2 with 5 M NaOH, and 10 μL of the supernatant was mixed with 90 μL of "Na-S" buffer (Beckman). Subsequently, the samples were analyzed for alanine production on a Beckman 6300 Amino Acid Analyzer.

Titrations of the Enzyme with Amino Acids. All titrations with D- or L-alanine were performed at room temperature in 50 mM potassium phosphate, pH 7.0, except where otherwise indicated. Amino acids were added to final concentrations mentioned in the text. With phosphate or pyrophosphate buffers, the enzyme was dialyzed against potassium phosphate prior to usage, while with bis-Tris, CHES, or CAPS as buffer the enzyme was dialyzed against bis-Tris, as mentioned above.

CD Spectra. CD spectra in the UV−vis and far UV were recorded with a Jasco J-715 spectropolarimeter at room temperature in 50 mM potassium phosphate, pH 7.0, or in 25 mM sodium pyrophosphate/HCl, pH 9.0.

Analysis of the Cofactor. The cofactor content of the enzymes was analyzed by the HPLC method on a TosoHaas ODS-80T_m (4.6 mm × 25 cm), reverse-phase silica gel column as described by van Ophem et al. (*14*) using 0.1% TFA as an eluent. Denatured protein samples were kept at room temperature for 5−10 min prior to injection to ensure complete release of the cofactor from the enzyme.

DTNB Titration and Amino Acid Analysis of Cys. Titration of SH groups was done using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman's Reagent) as described (*5*). The number of reactive SH groups was determined by using a molar absorbance coefficient of 14 000 M^{-1} cm^{-1} for the nitrothiophenylate anion (*17*). To determine total Cys residues, oxidation with performic acid was performed as described (*18*) prior to hydrolysis and amino acid analysis as described above.

Formation of Pyruvate or D-Alanine from L-Alanine. Formation of pyruvate was followed at room temperature using 11 μM enzyme in a coupled enzyme assay as described previously (*2*). Time-dependent conversion of L-alanine into D-alanine ("racemase" activity) was performed at room temperature by incubating 9 μM enzyme with 20 mM L-alanine in 0.35 M potassium phosphate, pH 8.5. Samples were taken at several time points, and racemization was terminated by denaturing the enzyme for 5 min at 100 °C. After removing the denatured enzyme by centrifugation for 3 min at 16000*g*, the supernatant was analyzed for D-alanine. This analysis was performed at room temperature in 0.35 M potassium phosphate, pH 8.5, in the presence of 0.1 mM NADH and 10 units of lactic dehydrogenase. After recording any decrease at 340 nm due to the presence of pyruvate from L-alanine, 1 unit of D-amino acid oxidase and 1000 units of catalase (both from Sigma) were added. Subsequently, the reaction was monitored until no further decrease in absorbance could be detected. If necessary, extra NADH was added until all D-alanine had been converted.

Temperature Inactivation. Enzyme solutions were incubated in 50 mM potassium phosphate buffer, pH 7.0, for 10 min at temperatures ranging from 25 to 75 °C with 1−1.5 °C steps in the temperature range at which the inactivation occurred and 2−5 °C steps at other temperatures. The following protein concentrations were used: E177K, 8.9 mg/mL; wild-type, 0.03 mg or 3.0 mg/mL. Subsequently, samples were cooled on ice water, and denatured protein was removed by centrifugation for 3 min at 16000*g*. The remaining activity in the supernatant was measured, as described above; a T_{50} value, the temperature at which an enzyme was inactivated for 50%, was calculated.

β-Decarboxylation of D-Aspartate. E177K was incubated at room temperature with 1 mM D-aspartate in 50 mM bis-Tris/HCl, pH 7.0. At several time points, samples were denatured and analyzed as described above.

RESULTS

Verification of the Mutation. The mutation was verified by DNA sequencing and peptide mapping. Analyses of the tryptic digests of the E177K and of the wild-type enzyme were done under the same conditions. The peptide map showed only one different peak present in the chromatogram of E177K compared to the wild-type enzyme. Amino acid analysis of that peak revealed that this peptide, containing 2 Asx, 2 Thr, 1 Val, and 1 Lys, could be ascribed to residues 172−177, indicating that the mutation was correct.

Spectral Properties. The spectrum of E177K shows a major absorption maximum at 415 nm and a small shoulder in the 335 nm region (Figure 1), indicating that the enzyme is mainly in the PLP form. This was confirmed by cofactor analysis (*14*), which indicated that purified E177K contained less than 1% PMP of the total cofactor content.

Titration of E177K with D-alanine was a very slow process and required hours for partial conversion (Figure 1a); 20 h was required for the enzyme to be transaminated predominantly to the PMP form (85% as judged by cofactor analysis). In contrast, addition of D-alanine to wild-type enzyme results in an immediate increase in absorbance at 330 nm with a

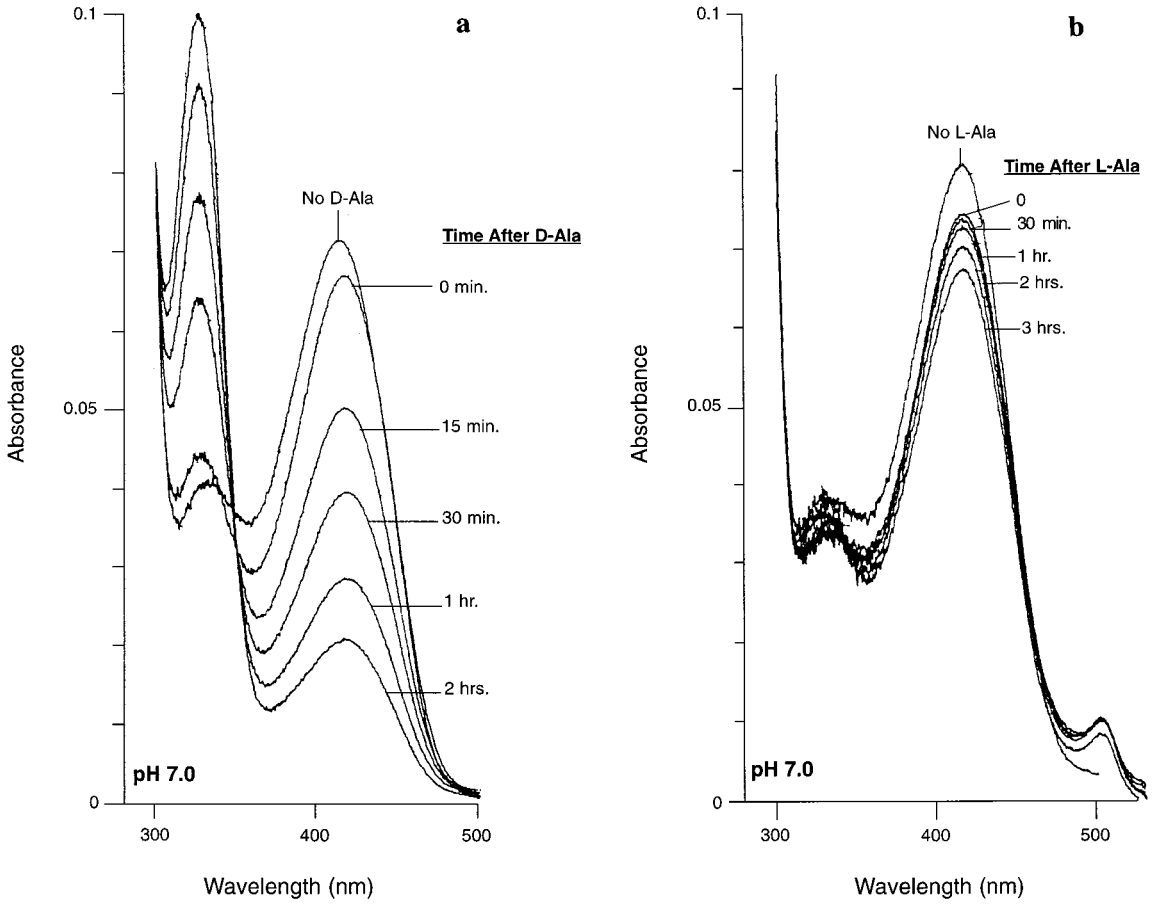

FIGURE 1: Time dependence of spectral changes of E177K with D- or L-alanine at pH 7.0. E177K (0.62 mg/mL) was mixed with 0.1 M D-alanine (a) or 0.1 M L-alanine (b) in 50 mM potassium phosphate, pH 7.0. At the indicated times, spectra were recorded. With L-alanine, the amplitude of the absorption band at about 500 nm remains constant with time; this band is not observed with D-alanine.

concomitant decrease at 415 nm, reflecting the rapid conversion of E−PLP into E−PMP. As with wild-type enzyme, the rate of conversion of D-alanine by E177K was dependent on the concentration of D-alanine added: 1 mM D-alanine changed the $A_{330\ nm}/A_{415\ nm}$ ratio from 0.50 to 0.62 in 1 h, while with 0.1 M concentrations this ratio changed to 3.27 in 1 h. For wild-type enzyme, the $A_{330\ nm}/A_{415\ nm}$ ratio changed immediately to 6.1 with 1 mM D-alanine. L-Alanine was also able to convert the PLP form into the PMP form (Figure 1b), although at a much slower rate than with D-alanine. Interestingly, with L-alanine but not with D-alanine, a small peak around 500 nm consistent with formation of a stable quinonoid intermediate was detected. Addition of 10 mM ethanolamine, which enhanced conversion in the K145Q mutant (*3*), had no effect on the spectral changes in E177K.

When D-alanine titrations were performed with E177K at higher pH values (at pH 9.0 both in 0.1 M sodium pyrophosphate/HCl as well as in 50 mM CHES/KOH and at pH 10.0 in 50 mM CAPS/KOH), a shift in the absorbance maximum was observed. Although the PLP peak retained a maximum at 415 nm, the PMP maximum shifted from 330 to 315 nm (Figure 2a). In contrast, such a shift was not observed with D-alanine with wild-type enzyme at these pH values (Figure 2b). The pK_a of the pyridinium nitrogen of free PLP in solution is 8.3. When this nitrogen is protonated, free PLP shows an absorption maximum at 330 nm, while the deprotonated form has a maximum of 315 nm (*19*). When PLP is bound in the wild-type form of the enzyme, this nitrogen is stabilized in the protonated state by Glu-177, so that it is likely that its pK_a would be even higher. When Glu-177 is changed to a Lys, however, this stabilization no longer exists. Thus, the E177K spectra indicate that this nitrogen is unprotonated at pH 9, whereas the wild-type enzyme still retains the proton on the pyridinium nitrogen.

When the mutant enzyme was titrated with 0.1 M D-glutamate or D-aspartate, the spectral changes were small; the decrease in $A_{415\ nm}$ was less than 10% after 1 h, whereas with 0.1 M D-alanine the change was almost 60% in the same time period. L-Glutamate or L-aspartate (0.1 M) failed to induce spectral changes.

CD Spectra. As with the wild-type enzyme, E177K showed a negative ellipticity at 415 nm. However, whereas wild-type and other mutants showed a positive peak near 280 nm, for the E177K enzyme this peak was near 270 nm (Figure 3) and it had a smaller extinction coefficient than that of wild-type enzyme. This result suggested a possible change in the microenvironment of the enzyme. Addition of 10 mM D-alanine in the presence or absence of 1 mM α-ketoglutarate resulted in only minor changes, taking hours for decreases in the peaks at 415 and 270 nm to be achieved (Figure 4). However, the peak in the 270 nm region did not shift toward another wavelength. At pH 9.0 in 25 mM sodium pyrophosphate, the peak around 270 nm did not shift. In addition, no peak could be detected around 330 or 315 nm with 10 mM D-alanine at this pH. In the far-UV, no large differences could be detected when comparing E177K and wild-type, either at pH 7.0 or at pH 9.0.

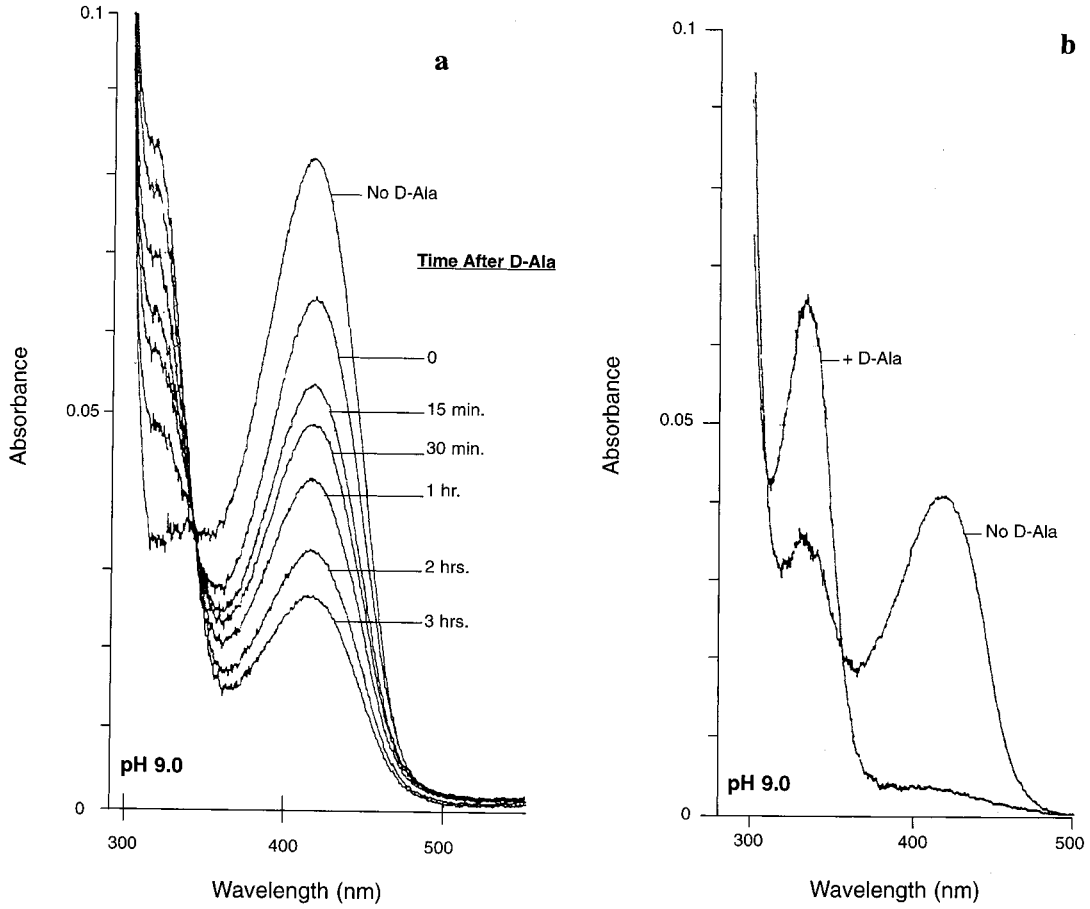

FIGURE 2: Time dependence of spectral changes of E177K and wild-type enzyme with D-alanine at pH 9.0. Enzyme (0.62 mg/mL) was mixed with 0.1 M D-alanine in 0.1 M sodium pyrophosphate/HCl, pH 9.0, and spectra were recorded at the indicated times. (a) E177K; (b) wild-type. With E177K, note the shift in absorbance at 330 nm at pH 7.0 (Figure 1) to 315 nm at pH 9.0.

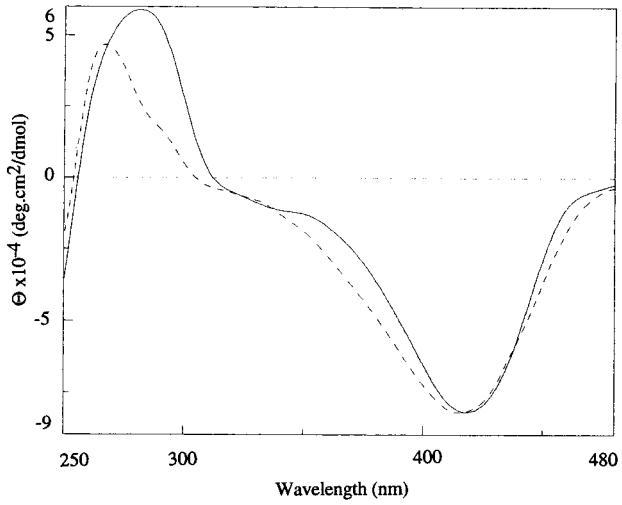

FIGURE 3: CD spectra of E177K and wild-type enzymes. CD spectra were recorded in 50 mM potassium phosphate, pH 7.0, at an enzyme concentration of 10 μM; E177K (dashed line), wild-type (solid line).

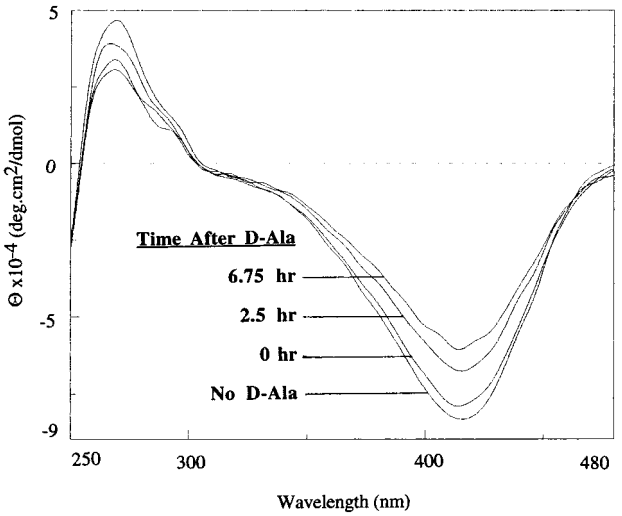

FIGURE 4: Time dependence of CD changes of E177K with D-alanine. E177K (10 μM) was titrated with 10 mM D-alanine in 50 mM potassium phosphate, pH 7.4. Spectra were recorded at the indicated times.

Titration of SH Groups. The E177K mutation dramatically increased the accessibility for the SH group reagent DTNB (Figure 5), yielding almost two SH groups per dimer after 1 h. In wild-type enzyme, this titration proceeds much slower (*5*), indicating a less accessible structure compared to E177K. Addition of 0.1 M D-alanine, which increased the accessibility of SH groups in the S146A mutant, but not of the wild-type (*8*), had no effect on the SH titration of the E177K enzyme.

DTNB titrations of the wild-type enzyme in the presence of 6 M guanidine show the presence of six SH groups per dimer. However, for E177K, a maximum of only four SH groups could be detected. Denaturation in 8 M urea yielded the same number. This result could indicate that in the monomer one of the SH groups is protected against reduction

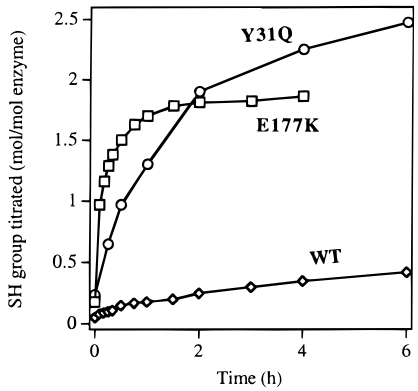

FIGURE 5: SH titration of E177K, Y31Q, and wild-type enzymes with DTNB. Wild-type or E177K enzyme (0.4 mg/mL) was incubated at room temperature in 0.1 M Tris/HCl + 2 mM EDTA, pH 7.4, in the presence of 1 mM DTNB. The Y31Q values are from ref 5. Given are the values per dimer.

with the TNB anion. Amino acid analysis of E177K after performic oxidation of Cys and calculating the expected/found ratio showed a value (0.90) almost as high as in wild-type (0.97), values also found for other residues. These results indicate that the correct number of Cys is present in E177K, but inaccesibility of one of the SH groups per subunit to the titrant in the E177K enzyme probably has taken place. The reason for this behavior is under investigation.

Crystal Structure. The overall structure of E177K is almost identical to that of the native enzyme in the PLP form (*20*). Neither the relative positions of the two domains in a subunit nor the monomer−monomer interactions in the dimer change are affected by the mutation. There are small differences in surface loops, but none of these are relevant to the chemical properties of the active site. The pyridine ring and the phosphate of the cofactor are in very similar positions in the two structures. Most of the interactions which hold the cofactor in place are still present.

The chemical environment below the cofactor did change, but in an unexpected manner. In the native enzyme, Glu-177, which is held in place by hydrogen bonds from Arg-138, forms a salt-bridge with atom N1 of the cofactor to stabilize its positive charge (Figure 6). Glu-177 was changed to a lysine to try to introduce a new titratable positive charge close to atom N1 of the cofactor to see its effect on the overall enzyme chemistry. The crystal structure of this new form of the enzyme shows the newly introduced lysine pointing away from the cofactor, most likely due to electrostatic repulsion, in the direction where Arg-138 used to be (Figure 7). Here it forms hydrogen bond contacts with the backbone carbonyl of Met-199, the side chain of Cys-142, and a possible weak interaction with Asp-136. Arg-138 has also moved and makes new hydrogen bond interactions with the backbone carbonyl of Asn-148 as well as the side chain of Cys-142. The latter is, most likely, the cysteine that becomes more reactive to DTNB titration. In addition, Arg-138 now points up toward the substrate binding site. There is a space with no electron density seen in the crystal structure close to the pyridinium nitrogen of the cofactor, with no new side chain from another part of the protein making an interaction. One cannot rule out the possibility of a disordered water molecule being bound at that position, although the electron density does not indicate one.

Consequently, even though this mutation did not succeed in introducing a positive charge close to atom N1 of the cofactor, it effectively eliminated the essentially permanent negative charge of Glu-177. This change in electrostatic environment may partially account for the biochemical results observed. The loss of this interaction most likely destabilizes the orientation of the cofactor, but it still remains tightly bound to the enzyme.

Kinetic Properties and Substrate Specificity for Transamination. Replacing Glu-177 by Lys had a very large effect on the specific activity, with a decrease from approximately 200 units/mg for wild-type to 0.15 unit/mg for the mutant. However, the mutation had little effect on the K_m for D-alanine, which was approximately 10 mM for D-alanine in the presence of 25 mM α-ketoglutarate (Table 2). The K_m for α-ketoglutarate in the presence of 200 mM D-alanine was approximately 5 mM (Table 2), which is higher than the 1 mM observed for wild type (*5*). L-Alanine (200 mM) could be utilized as a substrate, and the specific activity in its presence was 0.01 unit/mg. Both the D- as well as the L-isomers of aspartate and glutamate were tested as substrates in the presence of pyruvate. Both D-isomers produced approximately 16 nmol of alanine min^{-1} (mg of $protein^{-1}$), while with L-glutamate this value was around 12. With L-aspartate, alanine production was too low to be detected (less than 5 nmol of alanine produced min^{-1} mg^{-1}).

Pyruvate and D-Alanine Production from L-Alanine. E177K converted both D-alanine as well as L-alanine into pyruvate, although the amounts of pyruvate produced were low due to the diminished specific activities observed. To obtain a measurable amount of pyruvate from L-alanine, long incubations were required; after 2 h, 0.56 mol/mol of dimer was produced, while after 4 h the value was increased to 0.85. For wild-type enzyme, similar numbers were found after only a few minutes of incubation. E177K converted L-alanine into D-alanine, and the levels found [54 nmol of D-alanine produced min^{-1} (mg of protein)$^{-1}$] were more than 10-fold higher than those observed in wild-type (4 nmol produced min^{-1} mg^{-2}) (Table 2).

Optimization of the Racemase Activity. In this paper we use the term racemization for the conversion of L-alanine to D-alanine, although it is not a racemase activity as catalyzed by alanine racemase. Initially, racemization was measured in a coupled assay (*2*), which is useful for enzymes with low conversions of L- to D-alanine. However, when mutant enzymes with higher racemase activity were tested, the ongoing racemization during the assay (which takes up to 30 min) excluded a precise measurement of its rate. Therefore, detection of D-alanine has to be done after termination of the racemization as a point assay. Optimization of the racemase activity with E177K showed that at pH 7.0, racemization was only 10% of that found at pH 8.5, while in 50 mM EPPS, pH 8.5, the rate was approximately 65% of that observed in 0.35 M potassium phosphate. Doubling the enzyme concentration led to a 2-fold increase of the racemization rate, while varying the L-alanine concentration (10, 20, and 50 mM were tested) yielded almost identical rates. Addition of pyruvate, originally used to force the racemization toward D-alanine (*2*), resulted in somewhat lower (10%) activities.

Racemization Rates with Other Mutant Enzymes. The racemization method applied in this study revealed that all

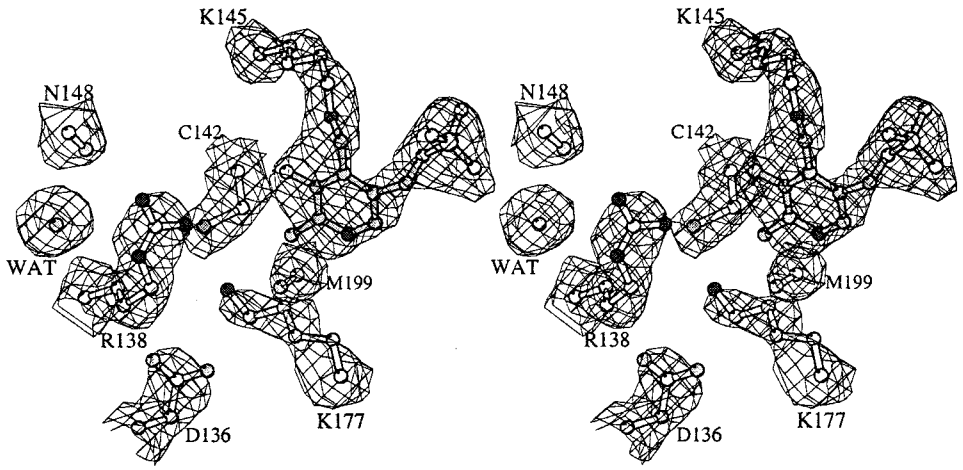

FIGURE 6: Stereoview of the electron density map of the active site of E177K. Electron density shown is an unbiased simulated annealing $2F_o - F_c$ omit map drawn at a 0.75 σ contour. Lys-177, Arg-138, and the cofactor were all omitted from the model for this map.

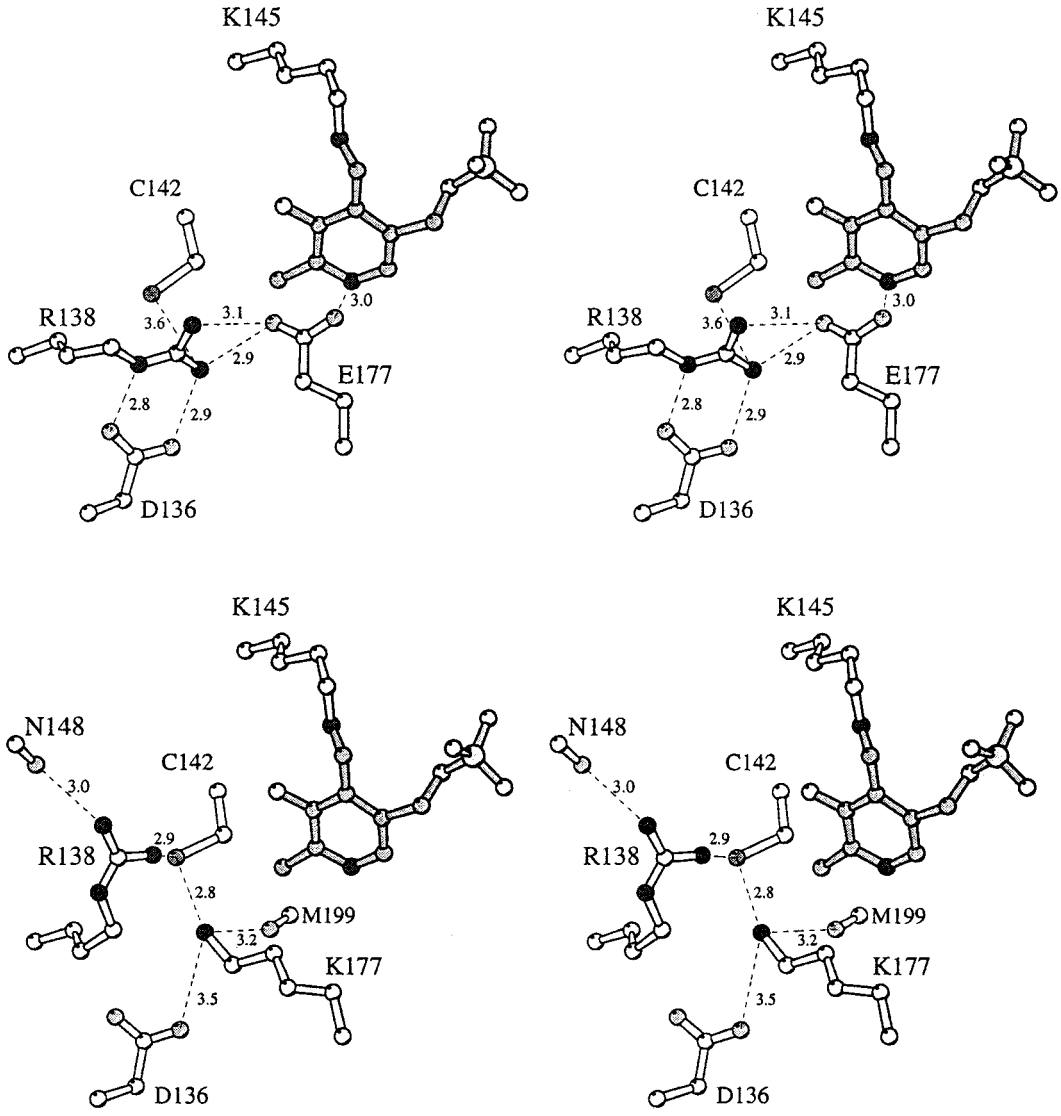

FIGURE 7: Stereoview of the structure of wild-type and E177K. Given are the residues interacting with Glu-177 (wild type, top) or Lys-177 (E177K, bottom).

mutant enzymes tested were able to convert L-alanine into D-alanine faster than wild-type with Y31Q showing the highest activities. However, we were not able to find a correlation between racemization and specific activity or between racemization and cofactor composition (Table 2). All racemization rates were significantly higher than with the wild-type enzyme.

Stability. Determination of the T_{50}, the temperature at which an enzyme was inactivated for 50% (*5*), for E177K yielded a value of 62.3 °C, which is higher than the values

Table 2: Comparison of Transaminase and Racemase Activities and Properties of Enzymes

enzyme	sp act. with D-Ala as substrate (units/mg)[a]	racemase act. with L-Ala as substrate (units/mg)[b]	% PMP[c]	K_m (D-Ala)[d] (mM)	K_m (α-Kg)[d] (mM)
wild-type	200	0.004	0.6	9.1	1.0
S180A	200	0.010	0.5	9.0	1.6
Y31Q	2.0	0.060	4.8	11.0	6.2
K145Q	0.02	0.049	51	nd[e]	nd
K145N	1.0	0.048	34	nd	nd
E177K	0.15	0.054	0.6	10.0	5.0

[a] Units/mg = μmol of pyruvate converted min^{-1} (mg of protein)$^{-1}$. [b] Units/mg = μmol of D-Ala produced min^{-1} (mg of protein)$^{-1}$; D-Ala produced from L-Ala was measured with D-amino acid oxidase and catalase. [c] % PMP was determined by an HPLC assay described by van Ophem et al. (*14*) with the balance as PLP. [d] Wild-type, S180A, and Y31Q were measured in 50 mM bis-Tris/HCl, pH 7.2 (*5*); E177K was measured in 0.1 M EPPS/KOH, pH 8.1. [e] nd = not determined.

for some other mutant enzymes and comparable to those found for wild-type. However, this value appeared to be dependent on the enzyme concentration used in the incubation. The wild-type enzyme at a concentration of 3.0 mg/mL, which is at least 10 times higher than used before (*5*), yielded a T_{50} of 68.5 °C, an indication that the enzyme concentration also affects the T_{50} value.

Wild-type enzyme gets slowly converted into its PMP form upon storage (*14*) in the absence of substrate. However, E177K remained in the PLP form even after a year of freezing/thawing cycles. Unlike wild-type, the activity of E177K was not stable upon storage at −80 °C and decreased approximately 50% after 6 months.

β-Decarboxylation of D-*Aspartate.* Under conditions optimal for β-decarboxylation of D-aspartate, wild-type enzyme produced approximately 370 nmol of alanine h^{-1} (mg of protein^{-1}), which is 0.003% of the transaminase activity (*21*). However, no alanine production could be observed with the E177K enzyme, even after 18 h of incubation.

DISCUSSION

The variety of residues interacting with the pyridinium nitrogen of the cofactor found in PLP-containing enzymes reflects their various roles. The presence of a negative charge is found in enzymes involved in releasing ketogroup containing compounds (*22*−*25*). Tryptophan synthase (*26*) and alanine racemase (*6*), however, which are not involved in such a reaction, have a neutral or positive charge near the pyridinium nitrogen, requiring other stabilization conditions for the intermediates to direct the reaction.

In D-amino acid transaminase, Glu-177 is involved in the interaction with the pyridinium nitrogen. Changing Glu-177 to Lys yielded some interesting changes in biochemical characteristics. The specific activity was reduced to a large extent, while the conversion of the PLP form to the PMP form in the presence of D-alanine is slow. The CD spectrum showed a shift from 280 to 270 nm, indicating a change in the chemical environment of PLP at the active site. The UV−vis titrations with D-alanine at pH 9 resulted in a peak around 315 nm rather than 330 nm.

The DTNB titration showed a large increase in reactivity to one Cys per monomer, indicating increased accessibility of one of the Cys residues. This was also found for the active site mutant Y31Q (Figure 5, *5*). Since Cys-142 is located close to the active site, this residue is the most likely candidate to be more reactive with DTNB in E177K or Y31Q. The crystal structure shows a larger open space close to this Cys, confirming this hypothesis.

The crystal structure shows significant changes in the active site which can explain the observed biochemical changes. The Lys-177 side chain now points toward Cys-142 instead of the pyridinium nitrogen, and Arg-138 moves to change the chemical environment of the active site, thereby becoming closer to Cys-142 as well. This may result in a more accessible active site and a more loosely bound cofactor. Arg-138 appears to be positioned on the edge of the substrate pocket, which may possibly explain the reduced reactivity with D-alanine due to increased steric/electrostatic hindrance. Glu-177 stabilizes the protonation of the pyridinium nitrogen in the wild-type enzyme. Its replacement, which led to some unaccounted space around that nitrogen, may have decreased the stability of the protonation of this nitrogen (the pK_a being around 8.5). Therefore, this results in an unbound PMP-like spectrum at pH 9.

The E177K mutation resulted in a much higher rate of L-alanine to D-alanine conversion, which has also been found for other mutant forms of D-amino acid transaminase (*3*−*5*). This increased activity can possibly be ascribed to the introduction of the positive charge, thereby resembling alanine racemase. But since the other mutant forms still containing the negatively charged Glu near the pyridinium nitrogen are also able to catalyze racemization (Table 2), it is more likely ascribed to a looser structure of the active site. The enzyme contains a carboxylate trap, which it uses to orient incoming substrates. The location of this trap and cofactor positions a D-amino acid such that its α-proton points to the catalytic base Lys-145, which is necessary for proton removal in the normal reaction pathway (*20*). Changing Glu-177 to a Lys may allow the cofactor−substrate complex to wobble to a larger degree than found in wild-type, accounting for the increased ability to racemize L-amino acids. As such, there appears to be no correlation between cofactor composition and either transaminase or racemase activity (Table 2).

In most of the PLP-containing enzymes whose structures have been solved, the pyridinium nitrogen of the cofactor forms a hydrogen bond with a negatively charged carboxyl side chain (*6*). It is assumed that this hydrogen bond stabilizes the carbanion intermediates of the reaction; the protonated form of the pyridine ring of the coenzyme then acts as an electron sink. However, in tryptophan synthase the neutral residue Ser-377 forms this hydrogen bond (*26*), while alanine racemase has a positively charged Arg-219 for this purpose (*6*). Replacing Glu by Lys in D-amino acid transaminase did not lead to a positive charge close to that nitrogen, but instead, the ε-NH_2 group was pushed aside. Therefore, it is likely that atom N1 of the cofactor is not stabilized, leading to a much more inactive enzyme as exemplified by the largely reduced specific activity and the slow turnover of D-alanine. However, it is noteworthy that the quinonoid intermediate is stabilized with the less efficient substrate, L-alanine.

In other PLP−enzymes, mutations near the pyridinium nitrogen site have been introduced. In L-aspartate aminotransferase, Asp-222 has been replaced by Ala, Asn, or

Glu (*22*, *23*), clearly showing the importance of a negative charge; although the Glu-mutant showed a reasonable activity, this was largely reduced in the other mutants. Similar results were found when Glu-274 was replaced with Ala in ornithine decarboxylase (*24*), and Asp-279 with Glu or Ala in aminolevulinate synthase (*25*). This study confirms the importance of the presence of the negative charge next to the pyridinium nitrogen most likely in order to ensure stabilization of the carbanion intermediate of the reaction.

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