Catalytic ability and stability of two recombinant mutants of D-amino acid transaminase involved in coenzyme binding

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Abstract

Of the major amino acid side chains that anchor pyridoxal 5'-phosphate at the coenzyme binding site of bacterial D-amino acid transaminase, two have been substituted using site-directed mutagenesis. Thus, Ser-180 was changed to an Ala (S180A) with little effect on enzyme activity, but replacement of Tyr-31 by Gln (Y31Q) led to 99% loss of activity. Titration of SH groups of the native Y31Q enzyme with DTNB proceeded much faster and to a greater extent than the corresponding titration for the native wild-type and S180A mutant enzymes. The stability of each mutant to denaturing agents such as urea or guanidine was similar, i.e., in their PLP forms, S180A and Y31Q lost 50% of their activities at a 5–15% lower concentration of urea or guanidine than did the wild-type enzyme. Upon removal of denaturing agent, significant activity was restored in the absence of added pyridoxal 5'-phosphate, but addition of thiols was required. In spite of its low activity, Y31Q was able to form the PMP form of the enzyme just as readily as the wild-type and the S180A enzymes in the presence of normal D-amino acid substrates. However, β -chloro-D-alanine was a much better substrate and inactivator of the Y31Q enzyme than it was for the wild-type or S180A enzymes, most likely because the Y31Q mutant formed the pyridoxamine 5-phosphate form more rapidly than the other two enzymes. The stereochemical fidelity of the Y31Q recombinant mutant enzyme was much less than that of the S180A and wild-type enzymes because racemase activity, i.e., conversion of L-alanine to D-alanine, was higher than for the wild-type or S180A mutant enzymes, perhaps because the coenzyme has more flexibility in this mutant enzyme.

Keywords: D-amino acid; mutagenesis; pyridoxal phosphate; transaminase

D-Amino acid transaminase catalyzes the transamination of all D-amino acids except D-proline into their corresponding α -keto acids. The physiological role of this enzyme in bacteria is the synthesis of D-alanine and D-glutamate, which are important constituents of the peptidoglycan layer of the bacterial cell wall. Because the enzyme is present exclusively in bacterial cells and has not been found in mammalian cells, it is a potential target for the development of antibiotics that act as suicide substrates (also known as mechanism-based inhibitors, k_{cat} inhibitors) for this enzyme (Soper & Manning, 1985). Transaminases for D- or L-amino acids contain pyridoxal 5'-phosphate as cofactor, which

is tightly bound by covalent attachment of its aldehyde function to a lysine residue as an internal aldime (Soper & Manning, 1985; Tanizawa et al. 1989a, 1989b) and also by noncovalent interactions of other parts of its pyridinium ring with amino acid side chains at the active site. The possibility that this rigidity may be involved in catalytic efficiency and stereochemical fidelity has not been studied to date and is addressed in this communication.

Transamination proceeds via a two-step mechanism; in the case of D-amino acid transaminase, D-alanine is first bound to PLP (Equation 1), thereby converting it via several intermediates into pyridoxamine phosphate with a concomitant release of pyruvate; this step is also referred to as the first half-reaction. In the second half-reaction (Equation 2), α -ketoglutarate binds to the PMP form of the enzyme and, upon formation of D-glutamate, the cofactor is converted back into its PLP-form.

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Abbreviations: PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; α -Kg, α -ketoglutarate; S180A and Y31Q, D-amino acid transaminase in which Ser-180 has been mutated to Ala and Tyr-31 to Gln, respectively; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid).

These half-reactions can be studied independently to provide mechanistic information on the enzyme. The conversion of E-PLP to E-PMP can easily be monitored, because binding of D-amino acids leads to a rapid spectral shift from 420 nm, due to the PLP internal aldimine structure, to 338 nm, due to the PMP (ketimine) form of the enzyme (Soper & Manning, 1985; Tanizawa et al., 1989a, 1989b). For D-amino acid transaminase, it has been demonstrated that the PLP is bound to Lys-145 (Tanizawa et al., 1989a, 1989b). Hence, mutations at this site led to a large reduction in activity (Futaki et al., 1990; Nishimura et al., 1991; Yoshimura et al., 1992; Bhatia et al., 1993a). However, the residual activity associated with these active site mutant enzymes was shown to be intrinsic, i.e., not due to contaminating wild-type enzyme. Lys-267, which is near the opening to the active site (Sugio et al., 1995), was shown to be responsible for this residual activity (Yoshimura et al., 1992). Because there is no internal aldimine in the Lys-145 mutant enzymes, the absorbance maximum at 420 nm due to this structure in the wild-type enzyme is shifted to 395 nm, which is attributed to the free aldehyde form of PLP. Addition of D-alanine to these mutant enzymes resulted in a shift of this absorbance to 420 nm, indicative of the external aldimine with substrate. Subsequently, the enzyme was slowly converted to the PMP form, as indicated by the appearance of the 338 nm peak (Futaki et al., 1990; Yoshimura et al., 1992). These active site mutant enzymes are referred to as "attenuated" because they catalyze the very slow turnover of substrates whose rates can be monitored by conventional spectrophotometry; measurement of the corresponding reactions by the wild-type enzyme are so rapid that specialized equipment is required.

Other amino acids that anchor the coenzyme at the active site (Fig. 1) have recently been identified by solving the structure of

the enzyme (Sugio et al., 1995). This structure provides information about the amino acid side chains that bind the cofactor PLP and permit a comparison with corresponding structures in the well-known L-aspartate transaminase. The objective of this manuscript is to assess the importance of two such amino acid side chains that bind pyridoxal 5'-phosphate and to ascertain any role that they may play in the overall mechanism of the enzyme. The two sites that were chosen for substitution, Ser-180 and Tyr-31, anchor opposite sides of the coenzyme. They were replaced by neutral amino acids that could not make strong interactions with the coenzyme so that their contribution to coenzyme rigidity, catalytic efficiency, and stereochemical fidelity could be made. The choice of substitutions was deliberately limited to neutral amino acids in order not to disrupt the coenzyme binding pocket with bulky or charged side chains that could weaken the binding of coenzyme.

Results

Activity of the enzymes

The specific activity of the S180A mutant was 210 units/mg, a value similar to that of the wild-type enzyme (Table 1). However, Y31Q had a considerably lower specific activity, i.e., 2.1 units/mg. For both mutants, the K_m values for D-alanine (measured in the presence of 20–25 mM α -Kg) were of the same order of magnitude found for the wild-type enzyme (Table 1), i.e., 11 mM and 9 mM for Y31Q and S180A, respectively, and 9 mM for the wild-type enzyme. The K_m values for α -Kg in the presence of 200 mM D-alanine were 0.95 and 1.56 mM for wild-type and S180A enzymes, respectively, and 6.2 mM for the Y31Q enzyme (Table 1). In earlier reports, the specific activity was measured in the presence of 5 mM α -Kg (Bhatia et al., 1993a, 1993b). However, the K_m values found here indicate that this concentration is not saturating. Therefore, the α -Kg concentration in the assay was increased to 20–25 mM to obtain the specific activities and K_m values for D-alanine given in Table 1.

Spectral properties of the mutant enzymes

The molar extinction coefficients of the purified enzymes, which are given in Table 1, are not very different from that found for the wild-type enzyme (spectrally or after amino acid analysis). The absorption spectrum of wild-type enzyme showed a major maximum at 415 nm and a minor maximum at 330 nm. For S180A, an almost identical spectrum was found. However, all Y31Q preparations showed a higher absorption at 330 nm than at 415 nm, compared to that found for the wild-type and S180A mutant (Table 1), suggesting that this mutant enzyme was partly in the PMP form when isolated. Direct cofactor analysis on HPLC (unpubl. results) confirmed that fresh Y31Q was indeed partly in the PMP form, whereas for fresh wild-type and S180A preparations, no PMP could be detected. Upon aging, the wild-type enzyme is slowly converted into the PMP form, e.g., the spectrum of a sample, which was stored at -80°C for several years, showed a much higher $A_{330\text{nm}}/A_{415\text{nm}}$ ratio (0.6–0.7) than a freshly prepared enzyme; cofactor analysis on HPLC also indicated a substantial amount of PMP. This phenomenon is currently under study. Analysis for PLP by the method of Wada and Snell (1961) confirmed the ratio of 1 cofactor/enzyme monomer for all three enzymes.

Fig. 1. Schematic active site of D-amino acid transaminase. From Sugio et al. (1995).

Table 1. Some properties of wild-type and mutant D-amino acid transaminase enzymes

Enzyme	Specific activity (D-Ala)	$\epsilon_{280\text{nm}}$ ($\text{M}^{-1}\text{cm}^{-1}$) ^a	$A_{330\text{nm}}/A_{415\text{nm}}$	Cofactor content ^b	PLP content ^c	K_m (D-Ala)	K_m (α -Kg)
				(per monomer)		(mM)	
Wild-type	210	57,000	0.32	1.1	1.06	9.1	1.0
S180A	210	58,200	0.28	1.0	1.08	9.0	1.6
Y31Q	2.1	58,800	0.4–0.7	1.1	1.14	11.0	6.2

^a ϵ was calculated from the $A_{280\text{nm}}^{0.1\%}$ as determined according to Van Iersel et al. (1985).

^b Cofactor content was calculated from the HPLC analysis and gives the value of PLP + PMP.

^c PLP content was determined by the method of Wada and Snell (1961). These values are precise to within 10%.

Titration of the wild-type enzyme with D-alanine resulted in a decrease in absorbance at 415 nm with a concomitant increase at 330 nm, due to the conversion of part of the enzyme to the PMP form (Martinez del Pozo et al., 1992). However, the rate at which this conversion took place varied, i.e., the wild type and S180A showed both a very rapid decrease in absorbance at 415 nm and corresponding increase at 330 nm; both changes occurred almost instantaneously and concomitantly, e.g., addition of 0.1 mM D-alanine to the wild type resulted in an instant decrease of 75% in the absorbance at 415 nm. With the Y31Q enzyme, the extent of conversion to the PMP form was similar, but it took about 5 min for completion, consistent with its slow catalytic turnover. When titrating the enzymes with D-alanine, large decreases in the 415 nm absorbance were found for the S180A enzyme and for the wild type (Fig. 2). For the Y31Q enzyme, analogous decreases at 415 nm required somewhat higher D-alanine concentrations. This result is in contrast to the large differences in the effects of β -chloro-D-alanine on the spectral properties of these enzymes (see below).

Enzyme stability

Because the substitutions in the recombinant D-amino acid transaminase were made at the active site, their stabilities were evaluated.

Wild-type D-amino acid transaminase is a very stable enzyme that can be kept in 50 mM potassium phosphate buffer, pH 7.0, at room temperature for up to a week without loss of activity. The S180A mutant enzyme has the same stability at room temperature. However, the Y31Q mutant enzyme is less stable at room temperature and loses approximately 50% of its original activity after 3–4 days. Addition of 1 mM D-alanine to the Y31Q enzyme in order to convert it to the PMP form improved its stability so that the enzyme could be kept in phosphate buffer at room temperature for a week without loss in activity. At 4 °C, each enzyme was very stable and the activity remained constant for several weeks. However, the absorption maximum of the Y31Q enzyme at 415 nm slowly decreased, whereas the value at 330 nm increased somewhat, e.g., the $A_{330\text{nm}}/A_{415\text{nm}}$ ratio was increased from approximately 0.5 to 0.8 in 2 weeks. The spectrum of the S180A mutant did not change significantly during storage at 4 °C; only a minor decrease at 415 nm (3–5%) with a concomitant increase at 330 nm was observed after 4 weeks. When stored at –80 °C, Y31Q lost some activity (10–20%) after 4 months, whereas S180A and wild-type enzyme did not lose activity during that period. Thus, replacement of Tyr-31 by Gln has a much greater effect on enzyme efficiency and stability than replacement of Ser-180 by Ala.

Thermostability

Wild-type D-amino acid transaminase is a thermostable enzyme (Soper & Manning, 1985; Merola et al., 1989). Earlier studies revealed that the wild-type enzyme was more stable than the mutants tested up to that time as judged from their T_{50} value, the temperature at which the enzyme is inactivated by 50% (Merola et al., 1989). In the present study, the mutant enzymes were found to be less thermostable than the wild-type enzyme as judged by lower T_{50} values (Table 2). The T_{50} values were dependent on the form of the coenzyme, i.e., the PLP form of enzymes were more thermostable than their PMP forms (Table 2). When determining the T_{50} for an aged wild-type enzyme with a $A_{330\text{nm}}/A_{415\text{nm}}$ ratio of about 1, a value of 63.7 °C was found, thereby fitting neatly between the T_{50} values for the PLP and the PMP form.

L-Alanine as a substrate

Previously we reported that L-alanine is a substrate, although a poor one, for the wild-type enzyme (Martinez del Pozo et al., 1989a, 1989b; Futaki et al., 1990). L-Alanine is also a substrate

Fig. 2. Titration of enzymes with D-alanine. Wild type (left), S180A (center), and Y31Q (right), each approximately 0.4 mg, were titrated with increasing concentrations D-alanine. D-Alanine concentrations (in μM) are given next to the titration curve.

Table 2. Thermostability (in °C) of D-amino acid transaminase enzymes

Enzyme	T_{50} (PLP-form)	T_{50} (PMP-form)
Wild-type	64.5	63.2
S180A	62.0	58.5
Y31Q	59.5	57.8

^a Enzymes were incubated for 10 min in 50 mM potassium phosphate buffer, pH 7.0, at T values varying from 25 to 75 °C. After cooling on icewater, the activity was measured and a T_{50} value, indicating the temperature at which an enzyme is inactivated for 50%, was calculated. The values are accurate to within approximately 0.5°.

for both the S180A and Y31Q enzymes (Table 3). The specific activities found in the presence of 5 mM α -Kg with 200 mM L-alanine were much lower than those found with 200 mM D-alanine (Table 3). Surprisingly, whereas higher activities were found with D-alanine as substrate in the presence of 20 mM α -Kg, with L-alanine, higher activities were obtained with 5 mM α -Kg. For purposes of comparison, in Table 3 the specific activities measured with 5 mM α -Kg are given. The ratio of the specific activities found with D-alanine compared with L-alanine indicated that Y31Q processed L-alanine in the transaminase reaction with the same efficiency as the wild-type and S180A enzymes; the ratios were 2.5–3 times higher for the latter than for the Y31Q enzyme.

The K_m value for L-alanine is greater than 200 mM for all three enzymes because a twofold increase in concentration led to a twofold increase in activity in the concentration range tested. The activities observed were not likely due to contamination of L-alanine with D-alanine, because the batch of L-alanine used contained less than 0.02% D-alanine. L-Alanine also had an effect on the spectra of the enzymes, i.e., addition of 1 mM L-alanine in all cases led to a small decrease in absorbance at 415 nm, as reported previously for the wild-type enzyme (Martinez del Pozo et al., 1989a). In contrast to D-alanine, which resulted in an almost instantaneous decrease in absorbance at 415 nm for the wild-type and S180A, this decrease with 1 mM L-alanine was slow for all three enzymes (Table 3). The Y31Q

enzyme showed the most rapid decrease in absorbance with L-alanine, although it was still slower than found with D-alanine. It was reported previously that D-amino acid transaminase is able to produce pyruvate from L-alanine (Martinez del Pozo et al., 1989a).

All three enzymes were also able to catalyze some degree of racemization, i.e., to produce D-alanine from L-alanine, but with substantial differences in efficiency (Table 3). The Y31Q mutant showed the highest "alanine racemase" activity, whereas with S180A, this activity was the lowest. In general, the data on L-alanine clearly indicate that this isomer is a better substrate for Y31Q than for the wild-type and S180A.

Inhibition by β -chloro-D-alanine

β -Chloro-D-alanine is an enzyme-activated inhibitor both for wild-type (Soper et al., 1977; Soper & Manning, 1985) as well as for the K145Q mutant (Yoshimura et al., 1992) of D-amino acid transaminase. For the Y31Q mutant enzyme, low concentrations of β -chloro-D-alanine (0.1–0.2 mM) led to rapid spectral changes and inhibition (Figs. 3, 4). After 10–30 s, the residual activity remained constant. A likely reason is that part of the chloro-D-alanine leads to inactivation (Fig. 4) and the remainder is hydrolyzed to products (see below). Hence, the extents of inactivation found with 0.5 and 1.0 mM concentrations were greater than those found with 0.1–0.2 mM β -chloro-D-alanine (Table 4). A significant difference was found between the mutant enzymes in the patterns of inhibition (Figs. 3, 4; Table 4). Whereas the S180A enzyme was only somewhat more sensitive toward β -chloro-D-alanine than the wild-type enzyme, the Y31Q enzyme was inactivated to a greater extent than the other two enzymes.

β -Chloro-D-alanine can also act as a substrate for the wild-type enzyme and also for the two mutant enzymes. For all three enzymes, a 1-mM concentration resulted in maximal activities, indicating that the K_m for β -chloro-D-alanine was in the micromolar range. These specific activities were compared with those

Table 3. Comparison of D-alanine and L-alanine as substrates for D-amino acid transaminase enzymes

Enzyme	Specific activity ^a (L-Ala)	Activity ^b D-Ala/L-Ala	Racemase activity ^c	ΔA_{415} ^d L-Ala	Pyruvate formed (nmol)
Wild-type	0.50	370	12	1.9	6.9
S180A	0.62	280	3	4.4	5.2
Y31Q	0.0065	125	34	10.5	7.3

^a Specific activity measured in presence of 5 mM α -Kg expressed as units/mg.

^b This ratio was calculated from the specific activity with D-alanine or L-alanine, each in the presence of 5 mM α -Kg.

^c Measured with L-alanine but no α -Kg, as described in the text (nmol NADH converted/min/mg).

^d Expressed as the decrease in absorbance at 415 nm/min and given as percent of the initial absorbance before adding 1 mM L-Ala.

Fig. 3. β -Chloro-D-alanine titration of wild type and Y31Q. Approximately 0.4 mg of wild type (right) or Y31Q (left) was titrated with 1 mM β -chloro-D-alanine in 50 mM potassium phosphate buffer, pH 7.0. Spectra were recorded before (thin line) and after (bold line) β -chloro-D-alanine addition.

Fig. 4. β -Chloro-D-alanine inhibition. Wild type (5 μ g), S180A (5 μ g), or Y31Q (325 μ g) was incubated with 0.5 mM β -chloro-D-alanine at 37 °C in 50 mM potassium phosphate buffer, pH 7.0. At the indicated times, a sample was taken, cooled on ice-water, and the remaining activity was measured. Control incubations did not contain β -chloro-D-alanine.

found under V_{max} conditions with 200 mM D-alanine, and a ratio giving the specific activity with 200 mM D-alanine compared to 1 mM β -chloro-D-alanine was calculated (Table 4). This ratio indicated that, for the Y31Q enzyme, the activity with D-alanine was only four times higher than that found with the suicide substrate, whereas for the wild-type and S180A, the corresponding ratio was nearly 100-fold higher. These data clearly indicate that the Y31Q mutant enzyme processes β -chloro-D-alanine much more efficiently than the wild-type or the S180A mutant enzyme.

Titration experiments with β -chloro-D-alanine indicated that the wild-type and S180A showed only minor spectral changes, which did not change during the 30-min incubations with this compound. However, the spectrum of Y31Q is remarkably influenced by β -chloro-D-alanine (Fig. 3). Thus, whereas a low concentration (1 mM) of the inhibitor resulted in only a slight shift of the 420 nm absorbance to 338 nm for the wild-type enzyme, the corresponding change in the Y31Q mutant enzyme was nearly complete (Fig. 3). Hence, in spite of its low catalytic

activity, the Y31Q mutant processes β -chloro-D-alanine much more efficiently and is consequently inactivated to a greater extent than either the wild-type or the S180A mutant enzymes. Possible reasons for this are discussed below.

Substrate specificity

Ser-180 forms part of a cavity into which the side-chain of the D-amino acid might fit (Fig. 1) (Sugio et al., 1995). Hence, a mutation of this residue could conceivably alter the substrate specificity of the enzyme. To evaluate this possibility, the wild-type and the S180A mutant enzymes were titrated with several D-amino acids and the spectral shifts were calculated and compared to the shift induced by D-alanine. For both enzymes, it was found that all D-amino acids were able to convert the PLP form to the PMP form at 1 mM concentrations Val, Leu, or Trp to about the same extent (95–99%) as observed with 1 mM D-alanine; Thr gave a somewhat smaller spectral shift (85–90%), whereas Gly gave little spectral shift (15%). With all concentrations tested, D-alanine produced immediate changes in the spectrum, but with the amino acids mentioned above, the changes were slower. D-Serine, however, was also able to produce an immediate change in the spectrum; with both enzymes there was a small peak at 495 nm, as has been detected previously (Martinez del Pozo et al., 1989b), due to a quinonoid intermediate. With Y31Q, only small spectral changes could be detected upon titration, giving shifts of 15–25% with 1 mM concentrations of the amino acids tested (except for Gly, which produced little spectral change).

Urea and guanidine denaturation

Wild-type and mutant enzymes were sensitive toward urea or guanidine, as judged by the loss of activity upon incubation for 1 h with various concentrations of these denaturing agents. A C_{50} value was calculated for the concentration of urea or guanidine at which 50% of the activity remained in the presence of these agents (Table 5). For enzymes in the PLP form, the wild-type enzyme was the most stable both toward urea as well as toward guanidine. The S180A mutant was more stable than Y31Q in the presence of urea, but the latter enzyme gave a higher C_{50} value in the presence of guanidine. In general, enzymes in the PMP form were less stable toward denaturation by urea or guanidine (Table 5) with one exception, i.e., Y31Q in its PMP form showed a higher C_{50} value in the presence of urea than in its PLP form.

The denaturation of the enzymes by urea or guanidine was time-dependent, i.e., when the remaining activity was measured after 20 h at room temperature instead of 1 h (Table 5), the C_{50} values were 1.1–1.3 M lower for PLP enzymes and 1.3–1.6 M lower for PMP enzymes in the case of urea, whereas in the presence of guanidine, the C_{50} value was 0.2–0.3 M lower for all coenzyme forms. For the wild-type enzyme, denaturation in urea was also determined by measuring the changes in absorbances at 270 and 286 nm, as described by Kellis et al. (1988). However, this treatment gave a rather scattered pattern when the ($\Delta A_{286} - \Delta A_{270}$) was plotted against the urea concentration. An approximate C_{50} value of 4.0 M was calculated; the value found was similar to that with the activity measurements. Hence, emphasis was placed on activity measurements, which gave more reproducible results.

Table 4. β -Chloro-D-alanine as inhibitor and as substrate of D-amino acid transaminase enzymes

Enzyme	Activity remaining with β -chloro-D-alanine (mM) ^a				Activity with D-Ala/ activity with β -Cl-D-Ala ^b
	0.1	0.2	0.5	1.0	
Wild-type	80	80	60	40	75
S180A	75	55	45	35	90
Y31Q	65	50	25	10	4.2

^a Enzymes were incubated with the indicated concentrations of β -chloro-D-alanine for 4 min. The percentages given are the values compared to the activity measured without β -chloro-D-alanine.

^b Ratio of activity measured with 200 mM D-alanine/activity measured with 1 mM β -chloro-D-alanine.

Table 5. Effect of urea and guanidine on D-amino acid transaminase enzymes^a

Enzyme	Urea concentration (M)		Guanidine concentration (M)	
	C ₅₀ (PLP form)	C ₅₀ (PMP form)	C ₅₀ (PLP form)	C ₅₀ (PMP form)
Wild-type	4.0	3.6	1.00	0.85
S180A	3.5	3.1	0.85	0.80
Y31Q	3.3	3.8	0.95	0.75

^a Enzymes were incubated in 50 mM potassium phosphate buffer, pH 7.0, containing urea concentrations from 0 to 8 M or guanidine concentrations from 0 to 4 M. After 1 h, the remaining activity was measured as described and a C₅₀ value, indicating the concentration at which an enzyme is inactivated for 50%, was calculated.

Refolding of denatured enzymes

After urea or guanidine denaturation, refolding of the enzymes in their PLP forms was achieved by dialysis against 50 mM potassium phosphate buffer, pH 7.0. However, the presence of an SH reagent, 0.01% β -mercaptoethanol, or at least 0.5 mM DTT, was essential for recovery of activity, i.e., of the order of 50–80%; in the absence of thiol, only about 10% activity could be restored. Increasing the concentration of DTT to 2 mM did not increase recovery of activity. The requirement for SH reagents for refolding was confirmed by recording the spectra of the enzyme preparations after dialysis, i.e., with buffers containing 0.5 mM DTT, the typical spectral pattern of absorbances at 415 nm and 330 nm were detected, but these spectral bands were absent in buffers without DTT.

The activity and spectral results above indicated that the denatured enzymes for the most part retained coenzyme. This conclusion is consistent with the finding that addition of PLP led to only a marginal restoration of activity (a maximum of 20%). When the enzymes in their PMP forms were tested for their ability to refold, little activity reappeared after dialysis and incubation at room temperature for several hours. This result clearly indicates that the PLP forms of the enzymes are more stable toward 8 M urea and 4 M guanidine than the PMP forms.

PLP-binding in the mutant enzymes

The studies on the refolding of the denatured enzymes in the presence of thiols after extensive dialysis suggest that the PLP remains bound to the enzyme because the aldehyde function of any residual PLP that remained after the extensive dialysis would have reacted with thiol. The high yields of activity are consistent with PLP remaining bound in an internal aldimine structure. Thus, the substitution of a Gln for Tyr-31 and an Ala for Ser-180 do not lead to an enhanced dissociation of the coenzyme.

Thiol titration with DTNB

D-Amino acid transaminase contains six free SH groups per dimer (Merola et al., 1989; Tanizawa et al., 1989a, 1989b); between five and six were also found for the three enzymes in the denatured state. Titration of the native, wild-type enzyme with DTNB showed that only one SH group is accessible over a 6-h time period (Merola et al., 1989). In the present study, these results were confirmed for both the native wild-type as well as

S180A enzymes (Fig. 5). In contrast, additional SH groups of the native Y31Q were more accessible to DTNB, because over the same period, 2.5 SH groups per enzyme molecule could be titrated. Because a cysteine is located near the active site (Sugio et al., 1995), this residue may be the one titrated with DTNB in the case of Y31Q. It has been found that, for other mutant enzymes, addition of 25 mM D-alanine to the incubation mixture led to a decrease in the number of titratable SH groups (Merola et al., 1989). However, with the enzymes tested here, that number did not change, showing that there are no differences for the accessibility to the SH groups for either the PLP or the PMP forms of the enzymes.

Titration with DTNB led to a decrease in enzyme activity. Y31Q showed the greatest inhibition; after 6 h, only 30–40% activity remained. For the same time period with S180A, 60–80% activity remained, whereas for the wild-type, 80–90% of the original activity was present. However, Merola et al. (1989) reported no inhibition of the wild-type thermostable enzyme after 2 h exposure to DTNB. Incubation of a thermolabile D-amino acid transaminase has also been reported to lead to inhibition of activity (Soper et al., 1985).

Fig. 5. DTNB titrations of SH groups of wild type, S180A, and Y31Q. For each of the three enzymes, 0.4 mg was incubated at room temperature in 0.1 M Tris/HCl, pH 7.0, and 2 mM EDTA. Values are expressed per dimer.

Discussion

The substitutions made at the coenzyme binding site of D-amino acid transaminase were deliberately chosen to be nondisruptive in this initial study because, in some native, wild-type PLP enzymes, the coenzyme is easily dissociated from the protein. We did not introduce radical or multiple substitutions in order to assess the possibility that the neutral substitutions that were made in the coenzyme binding pocket would be deleterious. For neither mutant enzyme did the substitution lead to loss of coenzyme either during purification or in any of the experiments.

The crystal structure analysis of D-amino acid transaminase revealed that Ser-180 forms part of a "hole" that looks like an entrance of a pocket in which the side-chain of the amino acid can fit (Fig. 1) (Sugio et al., 1995). Mutation of this residue to alanine, thereby making this hole somewhat more hydrophobic, did not affect the specific activity. However, it lowered the stability of the enzyme to some extent, because it became less thermostable and more sensitive toward denaturing agents such as urea and guanidine. No significant differences were found between the S180A mutant enzyme and the wild-type enzyme when comparing titration experiments performed with several D-amino acids. S180A was converted into its PMP form a little more rapidly upon D-alanine titration. In addition, the aliphatic and aromatic D-amino acids tested showed a slightly greater spectral shift with the S180A mutant, whereas with hydroxylated D-amino acids, this shift was somewhat smaller than with the wild-type. These observations can be ascribed to the more hydrophobic nature of the side-chain binding pocket, but the contribution of Ser-180 to this hole appears to be only minor.

The arrangement of three consecutive serine residues (Ser 179–181) in the wild-type enzyme anchoring the coenzyme (Fig. 1) (Sugio et al., 1995) as the analog of Trp-140 in L-aspartate transaminase is unusual but, as shown in the present study, at least the middle serine is not essential for catalytic activity for the binding of the coenzyme to the protein or for stereochemical fidelity. Hence, a constellation comprising the three hydroxyl groups of Ser residues need not be invoked as a requirement for coenzyme orientation.

Even though the overall catalytic activity of the Y31Q mutant is decreased, its K_m value for D-alanine is unchanged, although the K_m for α -Kg is increased by about fivefold. Perhaps the increased mobility of the coenzyme in the mutant Y31Q leads to an increased K_m for keto acids, whereas the rigidity associated with the E-PLP form of the enzyme, which is dictated more by the internal aldimine structure and less by side-chain coenzyme interactions, leads to unchanged values for the K_m values for D-amino acid substrates for all three enzymes. The substitution of Tyr-31 by Gln also has significant effects on the activity and stereochemical reaction pathway of D-amino acid transaminase, as well as its susceptibility to inactivation by β -chloro-D-alanine. The absence of the hydrogen bond between the 3'-OH of the coenzyme and Tyr-31 in this recombinant mutant means that the coenzyme is held less rigidly compared to the wild-type enzyme. Such increased flexibility leads to more efficient formation of the PMP form of the enzyme in the presence of β -chloro-D-alanine and also results in decreased stereoselectivity, i.e., the increased "racemase" activity. These results are consistent with our earlier conclusions on the active site K145Q mutant enzyme (Futaki et al., 1990). It appears that the rigidity of the coenzyme

at the active site is required for catalytic efficiency, reaction specificity, and stereochemical fidelity.

The effects of β -chloro-D-alanine as a substrate, inhibitor, and modulator of spectral behavior of the Y31Q enzyme is much different than would have been predicted from the other properties of this mutant. Its slow turnover with normal substrates such as D-alanine is in contrast to the greater inhibitory effect of β -chloro-D-alanine on the Y31Q mutant compared to the wild-type and S180A mutant enzymes. The much greater spectral shift from 415 nm (E-PLP) to 330 nm (E-PMP) catalyzed by Y31Q might explain the greater inhibition of this mutant by this mechanism-based inhibitor, i.e., more turnover leads to greater inhibition. A tentative mechanism has been proposed for the β -elimination and catalytic inactivation of D-amino acid transaminase by β -chloro-D-alanine to generate an intermediate α -aminoacrylate Schiff base with the coenzyme (Manning et al., 1974; Soper et al., 1985). It was concluded that the elimination of the β -halide ion was probably not rate-limiting, that the α -proton was rapidly abstracted, and that the processing of the α -aminoacrylate intermediate is the rate-limiting step. The results of this study present evidence for that proposal and further show that the rapid spectral shift of E-PLP to E-PMP by β -chloro-D-alanine for the Y31Q enzyme and its subsequent rapid inhibition mutant might be due to the more flexible environment for the PMP coenzyme.

The role of SH reagents in promoting maximum restoration of activity after exposure to urea or guanidine may be due to maintaining the free SH groups of the enzyme in the reduced state during the process of refolding by preventing formation of disulfide bridges, which the native wild-type enzyme does not possess. The lack of a requirement for PLP may indicate that the coenzyme stays bound to both the wild-type and the S180A and the Y31Q mutant enzymes either by the aldimine linkage to Lys-145 at the active site or in another form. Yoshimura et al. (1994) have made a similar observation for L-aspartate transaminase from *Bacillus* sp. YM-2. In the PMP form, the coenzyme is not bound covalently to the protein, but is bound by its functional groups to the side chains of amino acids in the active site. The finding that very little activity was regained after refolding implies that it is necessary that the cofactor must be firmly bound to the enzyme for refolding to its original state. The anchoring interactions between PMP and the amino acids are most likely too weak to bind the cofactor to the unfolded enzyme. Studies on refolding of other mutant enzymes, i.e., Lys-145, in which the coenzyme is anchored by other residues (Lys-267), may confirm this conclusion.

In general, the studies in this manuscript complement the X-ray crystallographic results (Sugio et al., 1995) because the latter methodology provides an indication of the nature of the important amino acid side chains at the active site and elsewhere on the enzyme, and the site-directed mutagenesis studies provide a measure of their relative importance in the overall function of the enzyme.

Materials and methods

DNA, phage, bacterial strains, enzymes, and site-directed mutagenesis

These experimental procedures have been described previously (Merola et al., 1989; Tanizawa et al., 1989b). Oligonucleotides

were synthesized at Operon Technologies, Inc., Alameda, California. The mismatched bases located approximately in the center of the sequences are given in italics. The following oligonucleotides were used.

S180A: 5'-AAA-TAC-ATT-TGA-TGC-AGA-GCC-TTC-TGT

Y31Q: 5'-TTT-CAC-AAC-TTC-TTG-TAC-GCC-ATC-AAC.

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 (Merola et al., 1989; Tanizawa et al., 1989b).

Purification of the mutant enzymes

The recombinant mutant enzymes were purified to near homogeneity by slight modifications of the procedures employed for the wild-type enzyme (Merola et al., 1989; Futaki et al., 1990; Bhatia et al., 1993a), i.e., during purification, the heat treatment was performed at 48 °C and EDTA was omitted from the elution and dialysis buffers used for the DEAE-Sepharose columns without affecting the total activity. After the second DEAE-Sepharose step, the enzymes were usually about 99% pure and, if needed, as ascertained from SDS-PAGE gels, a gel filtration step was used to remove the final impurities. This procedure was performed on a Superose-12 column (Pharmacia) equilibrated with 0.1 M potassium phosphate buffer, pH 7.6, containing 50 μ M PLP and 0.01% β -mercaptoethanol; the enzyme was eluted at a flow of 0.4 mL/min. The purified enzymes gave one band on SDS-PAGE. To remove excess PLP, preparations were dialyzed extensively against 50 mM potassium phosphate buffer, pH 7.0, at 4–6 °C. This treatment led to an increase in specific activity, in accord with the observation that PLP added to the assay resulted in lower activities (Bhatia et al., 1993a).

Proteolytic digestion

To verify that the mutant enzymes contained the desired substitution, 1 mg of each enzyme was reduced and carboxymethylated, as described previously (Yoshimura et al., 1992). After dialysis against 0.1 M NH_4HCO_3 for 16 h, the proteins were digested for 24 h at 37 °C with 2% TPCK-treated trypsin (Sigma type XIII from bovine pancreas). After lyophilization, the samples were dissolved in 0.1% TFA and the peptides were separated on a Vydac Protein and Peptide C-18 column (0.46 $\times$ 15 cm) and eluted with a linear gradient from 0.1% TFA to 0.1% TFA/80% acetonitrile (20–30% in 1 h for the S180A-peptide fragment, 10–30% in 90 min for the Y31Q-peptide fragment) at a flow of 1 mL/min. Detection of peptides was accomplished at 215 nm. Only one peptide was different between the wild-type and the mutant enzymes. Amino acid analysis of the mutant peptide was performed on a Beckman 6300 amino acid analyzer after hydrolysis in 6 N HCl for 18 h at 120 °C. Confirmation of the mutation of the S180A tryptic fragment was also performed by sequence analysis at the Rockefeller University Sequence Facility.

Enzyme activity

Enzyme activity was measured at room temperature by determining the rate of conversion of NADH to NAD at 340 nm in

0.1 M bis-Tris-HCl, pH 7.2, with a coupled assay using 10 units of lactic dehydrogenase (Sigma) in the presence of 200 mM D-alanine, 20 mM α -Kg, and 0.1 mM NADH. For the Y31Q recombinant mutant, the α -Kg concentration was increased to 25 mM due to its higher K_m value for this substrate (see the Results). Specific activities were calculated using a molar absorption coefficient of 6,220 $\text{M}^{-1} \text{cm}^{-1}$ for NADH at 340 nm (Dawson et al., 1986). One unit of enzyme activity is defined as the amount needed for the conversion of 1 μ mol NADH to NAD/min and corresponds to the conversion of 1 μ mol pyruvate derived from D-alanine to lactate. Specific activity is defined as units per milligram of protein. When testing L-alanine or β -chloro-D-alanine as substrates, these compounds replaced D-alanine in the assay mixture and, if necessary, the α -Kg concentration was altered, as indicated in the Results. Protein concentrations were determined by using the molar extinction coefficients at 280 nm given in Table 1. These values were calculated by applying the method described by Van Iersel et al. (1985).

Analysis of the cofactor

The cofactor content of the enzymes was analyzed by an HPLC method using a Toso Haas ODS 80 or ODS-120 column (unpubl.) and also by the method of Wada and Snell (1961).

Formation of pyruvate or D-alanine from L-alanine

The formation of pyruvate from D-alanine by the lactate dehydrogenase-coupled assay (transaminase activity) and the formation of D-alanine from L-alanine by the D-amino acid oxidase assay racemase activity were recorded at room temperature as described previously (Martinez del Pozo et al., 1989a). However, FAD was not included in the latter assay, because no differences in activity were detected in the presence or in the absence of this compound.

Substrate specificity

Approximately 0.4 mg of enzyme in 50 mM potassium phosphate buffer, pH 7.0, was titrated with 0.01, 0.1, and 1.0 mM concentrations of glycine and the following D-amino acids: alanine, valine, leucine, serine, threonine, and tryptophan. From the spectral changes, an $A_{415\text{nm}}/A_{330\text{nm}}$ ratio was calculated and these values were compared to those induced by similar concentrations of D-alanine, which were set at 100%.

Preparation of the PMP form of the enzymes

The PMP form of each enzyme was prepared by incubating each (wild type, 2 mg; S180A, 2 mg; Y31Q, 5 mg) with 0.1 M D-alanine in 50 mM potassium phosphate buffer, pH 7.0, for 30 min at room temperature. The formation of the PMP forms was verified by recording spectra before and after D-alanine addition. Excess D-alanine was removed either by extensive dialysis against 50 mM potassium phosphate, pH 7.0, for 16–18 h at 4–6 °C, or by desalting with 50 mM potassium phosphate buffer, pH 7.0, on a Hi-Trap column (Pharmacia) equilibrated with the same buffer.

Heat inactivation

Enzyme solutions (wildtype, 3–5 μg ; S180A, 3–5 μg ; Y31Q, 40–60 μg) 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 in which the inactivation occurred and 2–5 °C steps at other temperatures. Subsequently, samples were cooled in ice water and the remaining activity was measured, as described above. From the data thus obtained, a T_{50} value, i.e., the temperature at which an enzyme was inactivated by 50%, was calculated.

Urea and guanidine denaturation

An appropriate amount of each enzyme (wild type, 20–30 μg ; S180A, 20–30 μg ; Y31Q, 200–300 μg) was incubated in urea at concentrations ranging from 0 to 8 M, or in guanidine ranging from 0 to 4 M, in 50 mM potassium phosphate buffer, pH 7.0. After 1 h and 20 h at room temperature, the remaining enzyme activities were measured. Controls were performed to ensure that the lactic dehydrogenase used in the assay was still active.

For refolding experiments, enzymes were first incubated in 8 M urea or 4 M guanidine in 50 mM potassium phosphate buffer, pH 7.0, for 1 h at room temperature. Such treatment led to complete inactivation of the enzymes. After dialysis for 2 h at 4–6 °C against 1 L (1,000-fold excess) of 50 mM potassium phosphate buffer, pH 7.0, containing the additions described in the Results, refolding studies were performed at room temperature by measuring the reappearance of activity with time. Spectra were recorded at approximately the same times that the activities were measured.

DTNB titrations

Treatment with DTNB was performed on 0.4–0.5 mg of enzyme (both native as well as denatured in the presence of 6 M guanidine in 0.1 M Tris/HCl + 2 mM EDTA, pH 7.6) and the reaction with SH groups at 412 nm was followed periodically (Merola et al., 1989); the number of SH groups that had reacted was determined by using a molar absorbance coefficient of 14,000 $\text{M}^{-1} \text{cm}^{-1}$ for the nitrothiophenylate anion (Collier, 1973). When activity measurements were made, samples were removed immediately after recording the $A_{412\text{nm}}$ and assayed as described above.

β -Chloro-D-alanine as inhibitor

Enzymes (wild type, 5–6 μg ; S180A, 5–6 μg ; Y31Q, 250–300 μg) were incubated with various concentrations of β -chloro-D-alanine in 50 mM potassium phosphate buffer, pH 7.0, at 37 °C in a final volume of 0.5 mL. At the times indicated, 30 μL samples were removed and immediately cooled to 0 °C in ice-water to terminate the reaction. The remaining activity was recorded by assaying 25 μL of the sample, as described above.

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