Substrate Inhibition of D-Amino Acid Transaminase and Protection by Salts and by Reduced Nicotinamide Adenine Dinucleotide: Isolation and Initial Characterization of a Pyridoxo Intermediate Related to Inactivation†

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Received November 19, 1997

ABSTRACT: D-Amino acid transaminase, a pyridoxal phosphate (PLP) enzyme, is inactivated by its natural substrate, D-alanine, concomitant with its α-decarboxylation [Martinez del Pozo, A., Yoshimura, T., Bhatia, M. B., Futaki, S., Manning, J. M., Ringe, D., and Soda, K. (1992) *Biochemistry 31,* 6018−6023; Bhatia, M. B., Martinez del Pozo, A., Ringe, D., Yoshimura, T., Soda, K., and Manning, J. M. (1993) *J. Biol. Chem. 268,* 17687−17694]. β-Decarboxylation of D-aspartate to D-alanine leads also to this inactivation [Jones, W. M., van Ophem, P. W., Pospischil, M. A., Ringe, D., Petsko, G., Soda, K., and Manning, J. M. (1996) *Protein Sci. 5,* 2545−2551]. Using a high-performance liquid chromatography-based method for the determination of pyridoxo cofactors, we detected a new intermediate closely related to the inactivation by D-alanine; its formation occurred at the same rate as the inactivation and upon reactivation it reverted to PLP. Conditions were found under which it was characterized by ultraviolet−visible spectral analysis and mass spectroscopy; it is a pyridoxamine phosphate-like compound with a C_2 fragment derived from the substrate attached to the C′-4 of the pyridinium ring and it has a molecular mass of 306 consistent with this structure. In the presence of D-serine, slow accumulation of a quinonoid intermediate is also related to inactivation. The inactivation can be prevented by salts, which possibly stabilize the protonated aldimine coenzyme complex. The reduced cofactor, nicotinamide adenine dinucleotide, prevents D-aspartate-induced inactivation. Both of these events also are related to formation of the novel intermediate.

D-Amino acid transaminase catalyzes the transamination of D-amino acids, which are important constituents of the peptidoglycan layer of the bacterial cell wall (*1*). Transaminases for D- or L-amino acids contain pyridoxal 5′-phosphate (PLP)[1] as cofactor, which is tightly bound both by noncovalent interactions and also covalently to a lysine residue in the active site (*2*−*4*). Recently, the crystal structure of D-amino acid transaminase has revealed the various interactions of PLP in the active site (*5*).

The transamination proceeds via a two-step mechanism, in which D-alanine is first bound to the PLP and is converted via a quinonoid intermediate to pyridoxamine phosphate (PMP) with a concomitant release of pyruvate. In the second step, α-ketoglutarate binds to the PMP and, upon formation of D-glutamate, the cofactor is converted back into its PLP form. The conversion of the PLP form to the PMP form can easily be monitored since binding of D-amino acids leads to a rapid spectral shift from 415 nm, representing the PLP internal aldimine structure, to 332 nm, which corresponds to the PMP form (ketimine) of the enzyme (*3, 6*).

D-Alanine is an excellent substrate when used together with α-keto acid cosubstrates in short incubations with dilute enzyme concentrations, which are conditions occurring in kinetic assays. However, the enzyme becomes slowly inactivated when exposed to D-alanine alone (in the absence of α-keto acids) under conditions found at *equilibrium* (*7, 8*); i.e., substrates, intermediates and products coexisting with enzyme for several hours. Inactivation occurs concomitant with α-decarboxylation and is dependent on the type and concentration of the buffer used in the incubations (*7, 8*). Proportional to inactivation is the formation of a quinonoid intermediate, absorbing at 493 nm, especially with D-serine as substrate (*6*). At acidic pHs acetaldehyde or a closely related aldehyde is released together with NH_3, and the original spectum of the PLP form is restored together with full activity. In this study we investigate the effect of the buffers in more detail and demonstrate that the inactivation is influenced by the presence of salts, which affect the production of pyruvate from D-alanine. To gain more information on the nature of the intermediate fragment attached to the coenzyme, we have developed an HPLC-

† This work was supported by NSF Grant MCB 94-04332.

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine phosphate; PNP, pyridoxine phosphate; PXP, inactivated pyridoxo cofactor; LDH, lactate dehydrogenase; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; TFA, trifluoroacetic acid; CID, collision-induced decomposition.

S0006-2960(97)02842-0 CCC: $15.00
Published on Web 02/12/1998

based method to purify it and we have used mass spectroscopy and spectral analysis to determine a probable structure of the coenzyme-linked product derived from D-alanine. Furthermore, we have quantified the amounts of this intermediate and find a strong correlation between these values and the degree of inactivation of the enzyme. In addition, this correlation extended to the protection by NADH in the D-aspartate-induced inactivation (*9*) mediated by D-alanine. Finally, the use of the slow D-serine-induced inactivation has permitted us to propose an overall scheme for the substrate-induced inactivation, the spectral changes, the effects of NADH, and the formation of the novel intermediate.

EXPERIMENTAL PROCEDURES

Isolation and Purification of D*-Amino Acid Transaminase.* D-Amino acid transaminase was isolated and purified as described previously (*10, 11*). A final gel filtration on a Superose-12 column was sometimes performed using 0.1 M potassium phosphate, pH 7.3, as elution buffer instead of the buffer used for the purification, which contained PLP and 2-mercaptoethanol. To routinely remove excess PLP and 2-mercaptoethanol, the enzyme was extensively dialyzed against 50 mM bis-Tris/HCl, pH 7.0, or 50 mM potassium phosphate, pH 7.0.

Enzyme Activity. Activity was routinely measured at room temperature in a coupled assay with 10 units of lactate dehydrogenase (LDH; Sigma) in the presence of 0.2 M D-alanine, 0.1 mM NADH, and 25 mM α-ketoglutarate in 0.1 M EPPS/KOH, pH 8.1, the optimum pH for this assay (see also below). The concentration of α-ketoglutarate (5 mM) used to measure the activity of the wild-type enzyme (*8*) was found to be somewhat low, especially for the mutant enzymes (*11*). Therefore, its concentration was increased to 25 mM. Enzyme activities were calculated using a molar extinction coefficient at 340 nm of 6200 M^{-1} cm^{-1} (*12*). One unit of enzyme activity is defined as the amount needed for the conversion of 1 μmol NADH to NAD per minute, which corresponds to the conversion of 1 μmol of pyruvate derived from D-alanine to lactate. Protein concentrations were determined as described previously (*11*). The pH optimum was determined using the enzyme system described above and using 0.1 M concentrations of the following buffers: bis-Tris/HCl at pH 6.9 and 7.2; Tris/HCl from 7.2 to 8.5; HEPES/KOH from 7.2 to 8.5; EPPS/KOH from 7.3 to 8.7; Tricine/KOH from 7.4 to 8.8; CHES/KOH from 8.6 to 9.8; potassium phosphate from 6.9 to 8.1; all in 0.2−0.3 pH steps. The highest specific activities occurred at pH 8.1 with HEPES/KOH, EPPS/KOH, or Tricine/KOH. When salts were added to the assay mixture, the final pH was checked and adjusted, if necessary.

Inhibition by Substrates. Inhibition was studied using dilute (0.1 mg/mL) or relatively concentrated (2.5 mg/mL) enzyme solutions. All incubations were performed at room temperature in 50 mM bis-Tris/HCl, pH 7.0, except when otherwise indicated. The final D-alanine or D-serine concentration was 0.1 M, and D-aspartate was used at a final concentration of 1 mM. When salts were included in the incubation, the pH was adjusted to 7.0, if necessary, before the enzyme was added. LDH, which was in 3.2 M ammonium sulfate, was dialyzed extensively before use against 50 mM bis-Tris/HCl, pH 7.0. At the times indicated, samples were taken for cofactor analysis or activity measurements. For the latter, 10 μL samples of dilute incubations were mixed with 40 μL of 50 mM bis-Tris/HCl, pH 7.0, while for concentrated incubations, 10 μL samples were diluted in 490 μL of the same buffer. The activity remaining was assayed immediately after dilution as described above.

Pyruvate Production. Pyruvate production was measured after incubation of the enzyme (final concentration 2.5 mg/mL) with 0.1 M D-alanine. At the time points indicated, the reaction was terminated by chilling the incubation mix on ice−water for 1 min. The reaction products were separated from the enzyme by passing the mixture through a membrane filter (Amicon) with a cutoff of 10 000 Da. The filtrate was analyzed for pyruvate by measuring the decrease in absorbance at 340 nm at room temperature in 50 mM bis-Tris, pH 7.0, with 10 units of LDH in the presence of 0.1 mM NADH.

Protection by NADH. NADH was studied at concentrations varying from 0.1 to 0.3 mM in order to determine its efficiency as protector against substrate-induced enzyme inhibition, as described previously (*9*).

Preparation of the PMP Form. D-Amino acid transaminase (2.2 mg) was incubated with 0.1 M D-alanine in 50 mM potassium phosphate, pH 7.0, for 15 min. Removal of D-alanine was performed by changing the buffer to 50 mM bis-Tris/HCl, pH 7.0, (or 50 mM potassium phosphate, pH 7.0, when necessary) on a Hi-Trap (Pharmacia) desalting column. Subsequently, the enzyme fraction was concentrated as described above.

Cofactor Analysis. Samples (50 μL) from diluted incubations were denatured with 5 μL of 5% H_3PO_4, while denaturation of "concentrated" samples was achieved by mixing 10 μL samples with 50 μL of 67 mM potassium phosphate, pH 2.3. Analysis for PLP-related derivatives was performed using an HPLC method. Pyridoxine phosphate (PNP) was prepared by reduction of PLP with $NaBH_4$ as previously described for enzyme-bound PLP (*13*). Protein samples treated as described were kept at room temperature for 5−10 min prior to injection to ensure complete release of the cofactor from the enzyme. After the samples were centrifuged for 1 min at 16000*g*, samples (10−25 μL, corresponding to 100−300 pmol of cofactor) were injected onto a TosoHaas ODS-120T_m or ODS-80T_m (4.6 mm × 25 cm), reverse-phase silica gel column connected to a Shimadzu LC600 HPLC control system. The eluent was 67 mM potassium phosphate, pH 2.3, at a flow rate of 1.0 mL/min. A Shimadzu UV SPD-6AV detector set at 295 nm was employed and the peaks, which eluted over a 15 min period, were integrated with a Shimadzu Chromatopac Model CR501. Standard curves of peak areas as a function of the amount of coenzyme were prepared using commercially available PLP and PMP (Sigma) and the following integration values were obtained: PMP, 208 000 units/nmol; PLP, 133 000 units/nmol. The elution profiles were not significantly different for the two columns used. To attain accuracy and precision in the quantitative standardization of the cofactors, whose UV spectra vary considerably as a function of pH (*14*), enzyme solutions with a low buffer capacity (no higher than 100 mM phosphate buffer) were required. This precaution ensured that all coenzyme existed in a fully protonated state at the point of detection.

Purification of PXP, the Coenzyme Form Isolated from the Inactive Enzyme. Enzyme (10−20 mg/mL) was incubated with 0.1 M D-alanine and 10 mM α-ketoglutarate for 20 h at room temperature. For large-scale isolation of PXP, 0.1% trifluoroacetic acid (TFA) (pH 2.3) was used as an eluent, thereby replacing the phosphate buffer. After the solution was mixed with $^1/_{10}$ volume of 4% TFA and the denatured protein was removed by centrifugation for 3 min at 16000*g*, isolation of PXP was achieved by injecting 100 μL samples of the supernatant onto a TosoHaas ODS-80T_m HPLC column, equilibrated with 0.1% TFA and eluted with the same buffer at a flow of 1 mL/min. PXP, which eluted at approximately 8.7 mL, was lyophilized, dissolved in a minimal amount of ice-cold 0.1% TFA, and rechromatographed as described above to remove residual amounts of PLP. After the first chromatography step, all PXP solutions were kept at 0 °C on ice−water during handling, except during chromatography, which occurred at room temperature. After the last lyophilization the PXP solution was kept at −80 °C. For mass spectrometry, lyophilized samples were dissolved in water just prior to the analysis.

Mass Spectroscopy. Electrospray ionization mass spectra were obtained with a Finnigan-MAT TSO-700 triple-quadrupole mass spectrometer. Samples for negative ion spectra were electrosprayed from 1:1 water/acetonitrile mixtures at 100 μM concentrations and with the pH adjusted to 7.5 with ammonia. In the positive ion mode, spectra were taken on 50 μM samples in 50:50:5 water/methanol/acetic acid solvent. Tandem mass spectrometry in the phosphate loss mode was performed by scanning the first quadrupole of the TSQ-700 over the negative parent ion mass range of interest, effecting collision-induced decomposition at an argon pressure of 1.5 mTorr and a collision cell offset voltage of 10.2 V, and by setting the third quadrupole for constant transmission of $m/z = 79$ ions. By "standard" instrumentation conditions (see Results) we are referring to tuning the instument according to the manufacturer's recommendations, i.e., the voltages on the hardware elements along the ion path in the TSQ-700 are adjusted to maximize ion transmission. In our experience, under these conditions covalently bonded compounds show a small extent of fragmentation, typically less than 10%. By "much gentler conditions" we mean instrument settings where the potential differences between hardware elements in the preskimmer region were significantly reduced. Specifically, in the $m/z = 300$ region, we have reduced the potential on the heated capillary from −10 to −4.2 V, on the tube lens from −58 to −28 V, and on the entrance octapole from 5.0 to 0.2 V. Under these conditions fragmentation of ordinary covalently bonded compounds is negligible and adduct peaks are strongly enhanced.

Spectral Analysis of PXP. A PXP solution containing 87 nmol/mL (based on the correlation factor determined by HPLC; see Results section) in 50 μL was added to 20 mM potassium phosphate solutions at various pH values. Spectra from 500 to 220 nm were recorded at various pH values against water indicated in the text.

RESULTS

D-*Alanine Inhibition.* Expanding on an earlier observation that enzyme concentration played a role in D-alanine-induced inhibition, we have found that at increasing enzyme concentrations between 0.1 and 2.5 mg/mL there is a proportional inhibition with a linear relationship found when the logarithm of the relative activity was plotted against time (Figure 1), from which an inhibition rate was calculated. α-Ketoglutarate showed no inhibition but led to enhanced inhibition with D-alanine (Figure 2), as reported earlier (*7*). Replacing α-ketoglutarate with pyruvate gave a similar inhibition pattern. However, unlike α-ketoglutarate, pyruvate by itself was able to inactivate the enzyme slightly, i.e., 8% after 6 h with 10 mM pyruvate alone. This result was confirmed by cofactor analysis showing a small peak of an intermediate referred to as PXP (see below), while with α-ketoglutarate no such peak could be detected.

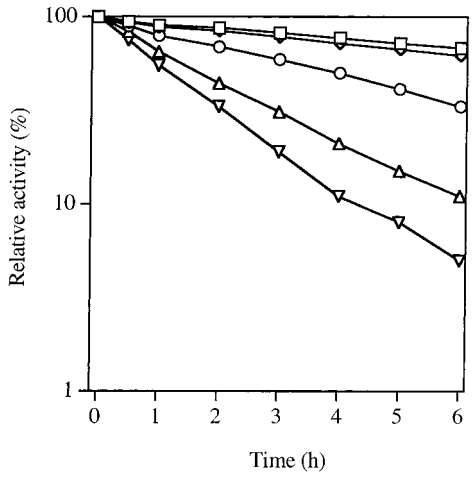


FIGURE 1: D-Alanine inhibition: effect of enzyme concentration. The following enzyme concentrations were incubated with 0.1 M D-alanine: 0.1 mg/mL (□), 0.2 mg/mL (◇), 0.5 mg/mL (○); 1.0 mg/mL (△), 2.5 mg/mL (▽). At the indicated time the activity was measured. Plotted is the logarithm of the relative activities vs time.

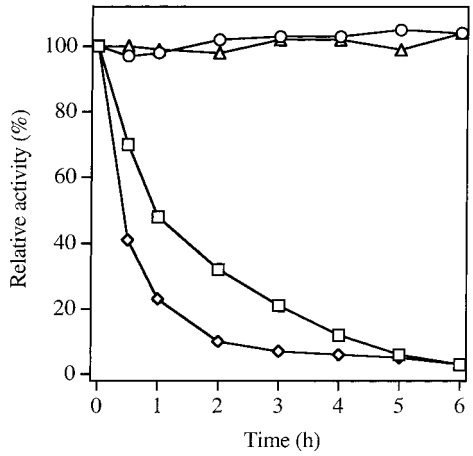


FIGURE 2: D-Alanine inhibition: effect of α-ketoglutarate. Enzyme (2.5 mg/mL) was incubated with 0.1 M D-alanine (□), 10 mM α-ketoglutarate (○), 0.1 M D-alanine + 10 mM α-ketoglutarate (◇), or no addition (△). At the indicated time the activity was measured.

Effect of Salts on the Activity. Increasing salt concentrations in the coupled assay led to a decrease in activity and the lowest activities were found in the presence of $MgCl_2$ (Figure 3). Similar concentrations of Na_2SO_4, which has an identical ionic strength, did not lead to a comparable level of inhibition. It is not likely that Cl^- ions alone are responsible for this inhibition since 0.1 and 0.2 M concentrations of $MgCl_2$ gave activities comparable to those with 0.2 and 0.4 M KCl or NaCl, respectively.

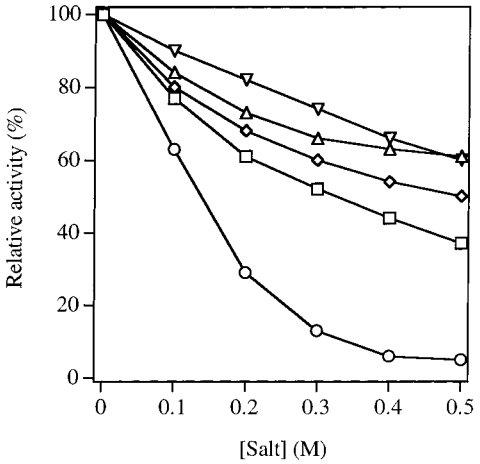


FIGURE 3: Effect of salts on the D-amino acid transaminase activity. The indicated concentration of salts was added to the assay mixture used for the coupled D-amino acid transaminase/lactate dehydrogenase assay. The following salts were tested: KCl or NaCl (□), NaOAc (▽), $MgCl_2$ (○), $MgSO_4$ (◇), and Na_2SO_4 (△).

Table 1: Effect of Salts on Inhibition of Activity by D-Alanine[a]

salt added	% inhibition by D-alanine: 2.5 mg/mL protein	% inhibition by D-alanine + α-ketoglutarate: 0.1 mg/mL protein	% inhibition by D-alanine + α-ketoglutarate: 2.5 mg/mL protein
no additions	40	85	70
0.1 M KCl/NaCl	18	80	50
0.2 M KCl	9	70	40
0.5 M KCl/NaCl	0	55	25
1.0 M KCl	0	nd[b]	13
0.1 M $MgCl_2$	10	nd	nd
0.05 M Na_2SO_4	7	nd	nd
0.25 M Na_2SO_4	0	nd	nd
0.064 M $(NH_4)_2SO_4$	0	nd	nd
50 mM phosphate, pH 7.0[c]	0	50	40

[a] Final concentrations of salts added to 50 mM bis-Tris/HCl are shown (final pH = 7.0). Incubations contained a final concentration of 0.1 M D-alanine and, if included, 10 mM α-ketoglutarate. Given is the calculated percent inhibition per hour as shown in Figure 1. [b] nd, not determined. [c] Potassium phosphate replacing bis-Tris as incubation buffer.

Effect of Salts on Inhibition. As shown in Table 1 for concentrated enzyme samples, salts are able to protect the enzyme from D-alanine-induced inhibition and increasing the salt concentration leads to an increased protection. In diluted samples, D-alanine inactivation was slight and addition of salts completely prevented inhibition. Inclusion of α-ketoglutarate with D-alanine showed a similar result.

D-Alanine Titrations. The protective effect of salt was also found when titrating the enzyme with D-alanine, indicating a possible influence on the conversion of the PLP into the PMP form. When titrations in 50 mM phosphate and 50 mM bis-Tris/HCl were compared, large differences were detected with identical concentrations of D-alanine (data not shown); i.e., in phosphate buffer the decrease at 415 nm and the concomitant increase at 330 nm were much larger than in bis-Tris. With increasing KCl concentrations in the presence of the bis-Tris buffer, an increasing conversion was found as indicated by the larger increase in the A_{330nm}/A_{415nm} ratio (data not shown).

Pyruvate Production. The amount of pyruvate formed was dependent on the nature of the buffer used (Figure 4). With

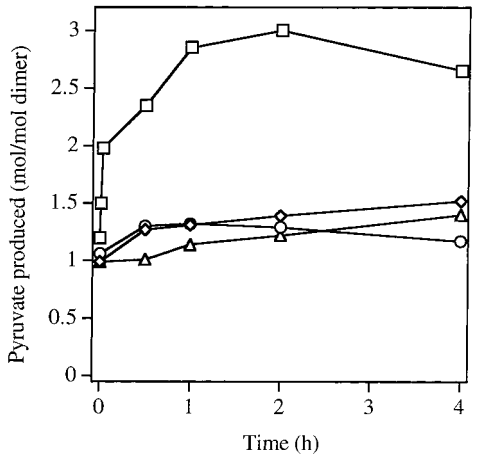


FIGURE 4: Pyruvate production during D-alanine inhibition. Enzyme (2.5 mg/mL) was incubated with 0.1 M D-alanine. At the indicated time the reaction was terminated by cooling on ice−water, and enzyme and reaction products were separated through a membrane filter. The filtrate was analyzed for pyruvate with NADH and lactate dehydrogenase. Tested were 50 mM bis-Tris, pH 7.0 (□); 50 mM bis-Tris + 0.2 M KCl, pH 7.0 (◇); 50 mM bis-Tris + 0.5 M KCl, pH 7.0 (○); and 50 mM potassium phosphate, pH 7.0 (△).

0.1 M Tris/HCl + 2 mM EDTA, pH 7.0, D-alanine is rapidly converted into pyruvate, yielding 2 mol of pyruvate/enzyme dimer initially (*7*). However, as a function of time during D-alanine inactivation, the amount of pyruvate produced per dimer decreased to 1 mol after 4 h (*7*). In this study, for 50 mM bis-Tris/HCl, pH 7.0, 2 mol of pyruvate/mol of enzyme was detected after the enzyme was incubated with D-alanine at room temperature for 1 min, while in 50 mM potassium phosphate, pH 7.0, only 1 mol of pyruvate/mol of enzyme could be detected. Shorter incubation times in bis-Tris/HCl led to lower pyruvate production, but even at the shortest time possible, incubation in bis-Tris/HCl still yielded 1.25 mol of pyruvate/mol of dimer. Over a period of several hours the pyruvate/enzyme ratio increased, reaching a maximum of 3 after 2 h in bis-Tris, while in phosphate buffers or in bis-Tris buffers supplemented with KCl, no maximum level was reached (Figure 4), indicating that pyruvate production proceeds during incubation with D-alanine. Thus, salts have a clear impact on the amount of pyruvate produced.

Effect of LDH and NADH. Since differences in the amount of pyruvate production can influence inhibition by D-alanine, the transaminase was incubated with LDH and NADH to remove pyruvate. With 0.1−10 mM concentrations of NADH and either dilute or concentrated enzyme, inhibition by D-alanine decreased but it could not be prevented (Figure 5). Only small differences were found comparing incubations with and without LDH/NADH in the presence of D-alanine and 10 mM α-ketoglutarate. This is likely due to the high levels of pyruvate, which were immediately produced upon addition of enzyme.

Inactivation of the PMP Form. This PMP form of the enzyme was prepared as described under Experimental Procedures. The coenzyme form was 95% PMP, as judged from HPLC analysis (see below); after incubation with 10 mM pyruvate in 50 mM bis-Tris, pH 7.0, very little inhibition could be detected.

Reactivation. D-Alanine-inactivated enzyme could be reactivated upon adjusting the pH to 5, although the yields

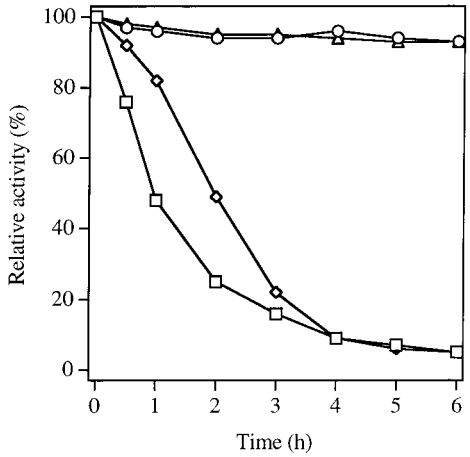


FIGURE 5: D-Alanine inhibition in the presence of NADH and lactate dehydrogenase. Enzyme (2.5 mg/mL) was incubated with 0.1 M D-alanine, 10 units of lactic dehydrogenase, and the following concentrations of NADH: 0 mM (□), 0.1 mM (◇), 1 mM (○), and 10 mM (△). At the indicated time the activity was measured.

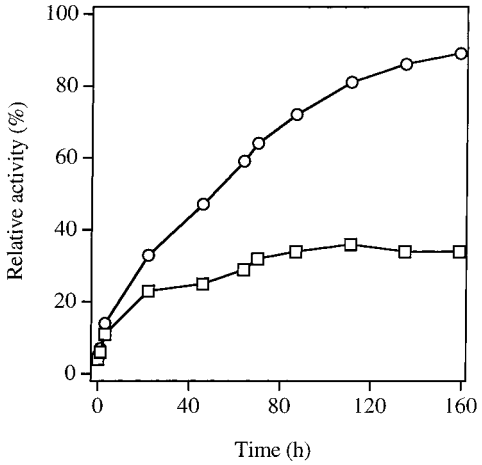
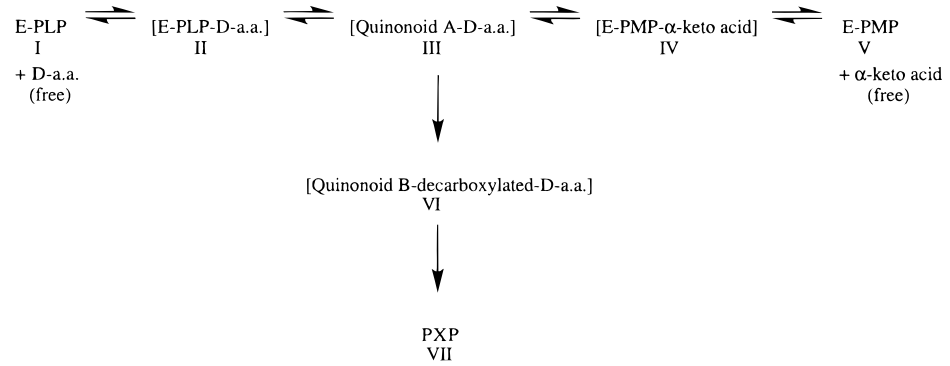


FIGURE 6: D-Alanine inhibition: reactivation and the effect of KCl. Enzyme (2.5 mg/mL) was inactivated with 0.1 M D-alanine and 10 mM α-ketoglutarate for 3 h. Substrates were removed on a Hi-Trap column with concomitant replacement of buffer using 50 mM bis-Tris/HCl, pH 7.0 (□) or 50 mM bis-Tris/HCl + 0.1 M KCl, pH 7.0 (○). At the indicated time activity was measured.

were not studied in the previous report (*7*). We have now systematically studied this behavior. When the enzyme was inactivated to the extent of 97% with D-alanine and α-ketoglutarate, and subsequently the pH was adjusted to 5.3, slow reactivation could be detected, but only to the extent of 15−20%. We suspected that enzyme denaturation was the cause due to the acidic conditions used to lower the pH. Therefore, the pH was changed on a Hi-Trap (Pharmacia) desalting column, thereby also removing the inactivating substrates. Lowering the pH alone is not required for reactivation, since phosphate buffers at pH 7.0 gave similar reactivation compared to pH 5.0. Salts play a role in reactivation, e.g., in 50 mM bis-Tris/HCl, pH 7.0, almost complete recovery was obtained in the buffer containing 0.1 M KCl, while only 40% of the original activity was recovered in its absence (Figure 6).

D-*Serine Inactivation*. D-Serine inhibits D-amino acid transaminase as reported previously (*6*). This inactivation is now shown to occur at the same rate as with D-alanine. However, unlike for D-alanine, the D-serine-mediated inactivation occurs with the appearance of an quinonoid intermediate with a high absorption at 492 nm (*6*). Assuming that this quinonoid might have similar features as the corresponding intermediate with D-alanine, we investigated the effect of salts on the occurrence of the quinonoid (Figure 7) in order to distinguish the changes at the wavelengths of interest since spectral changes with D-serine are relatively slow compared to those with D-alanine. As found with D-alanine, there was an immediate drop in absorbance at 415 nm with a concomitant increase at 335 nm upon addition of 10 mM D-serine in the absence of salt. But unlike with D-alanine, this process was quickly reversed and an absorbance at 492 nm appeared, as reported previously (*6*). We now report that these spectral changes are dependent on the presence of salts; i.e., the rates of increase at 415 nm, the concomitant decrease at 335 nm, and the appearance of the 492 nm peak varied with the salt concentration, with higher concentrations giving slower rates of change at all these wavelengths. In the absence of salts, the maximum absorbances at 415 and 492 nm are reached after approximately 1 min (*6*), and subsequently these absorbance decrease with a concomitant increase at 335 nm (Figure 7), probably due to the occurrence of the inactivated cofactor (see below). Increasing KCl concentration resulted in a general slowing of the spectral changes at 415 and 335 nm so that the decrease in the former and the increase in the latter as a function of time were not observed within 1 h. This effect is more readily discernible at 492 nm, where the absorption maximum of the quinonoid is nearly completely suppressed with increasing salt concentration. Our interpretation of these changes in reference to Scheme 1 is that salts decrease all species formed after intermediates I and II, i.e., the quinonoid form, the PMP form of the enzyme (intermediate IV or V),

Scheme 1: Pathway for D-Amino Acid-Induced Inactivation



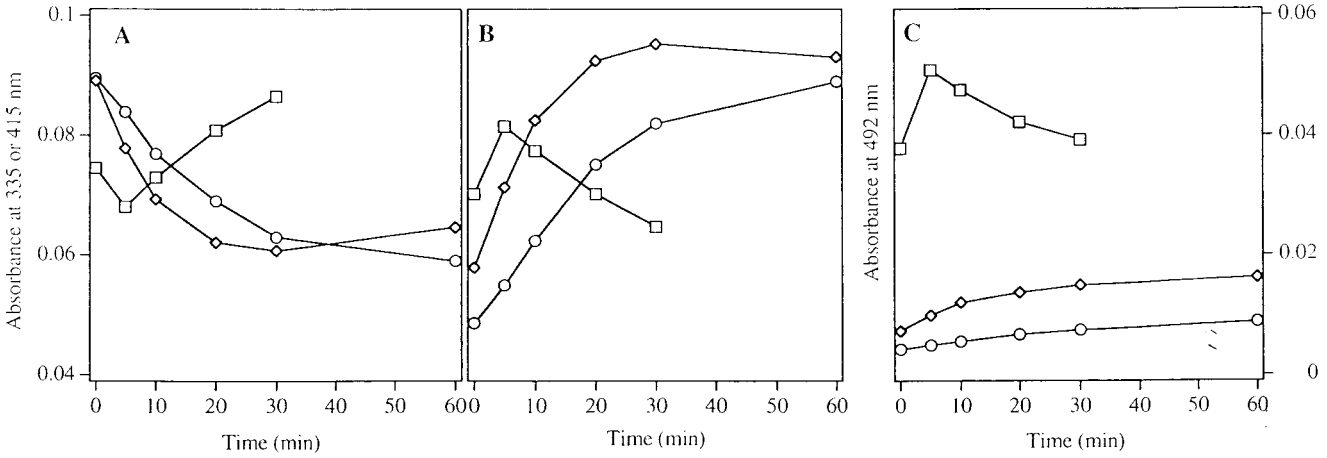


FIGURE 7: Titrations with D-serine. Enzyme (0.66 mg/mL) was titrated with 10 mM D-serine at room temperature at pH 7.0 in 50 mM bis-Tris/HCl supplemented with (□) 0 M KCl, (◇) 0.1 M KCl, or (○) 0.2 M KCl. Spectra were recorded at the indicated times, and given are the absorbances at 335 nm (panel A), 415 nm (panel B), and 492 nm (panel C). Before addition of D-serine the following absorbances were detected: $A_{335nm} = 0.036$, $A_{415nm} = 0.125$; and $A_{492nm} = 0.000$.

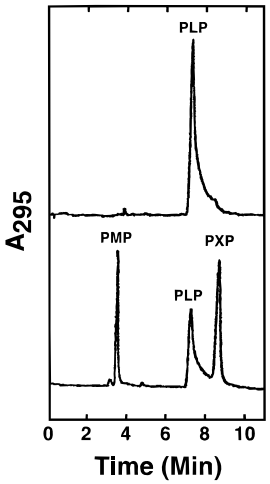


FIGURE 8: Cofactor analysis of D-amino acid transaminase. Enzyme was analyzed for cofactor content as described in the text before (upper panel) and 30 min after addition of 0.1 M D-alanine (lower panel).

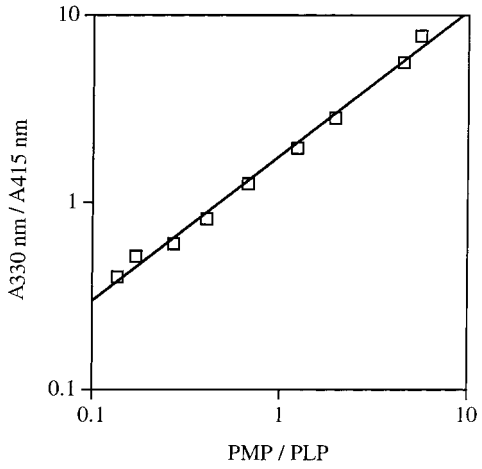


FIGURE 9: Correlation between cofactor ratio and spectral properties. The ratio of PMP/PLP was determined by HPLC analysis on enzyme samples containing PMP and PLP after addition of D-alanine. The absorption spectra of the same sample were recorded.

the formation of free pyruvate, as well as inactivation of the enzyme and PXP formation (see below). Replacing bis-Tris with 50 mM potassium phosphate, pH 7.0, gave spectral changes comparable to those observed with 50 mM bis-Tris + 0.1 M KCl, pH 7.0 (data not shown). However, just as with D-alanine (see above), the immediate decrease at 415 nm and the concomitant increase at 335 nm were larger in the phosphate buffer.

Coenzyme Composition of D*-Amino Acid Transaminase.* The HPLC analyses confirmed that freshly purified wild-type enzyme, like most other pyridoxal enzymes, had all of its coenzyme in the PLP form (Figure 8, upper panel). This result is consistent with the spectral properties of the enzyme since practically all of the absorbance is at 415 nm (see also Figure 9). It is also consistent with the chemical derivatization assay with phenylhydrazine, which indicated that 2 mol of the hydrazone derivative was found/mol of dimeric enzyme (*15*). The PLP coenzyme of the wild-type enzyme was converted very slowly into the PMP form in the absence of any D-amino acid. This phenomenon has also been observed for L-aspartate transaminase (*16*, *17*). Its origin is under study.

The HPLC assay was useful in determining the ratio of coenzyme forms in the enzyme as a function of their spectral properties, as the PLP-enzyme was titrated to the PMP form. The relationship is double-logarithmic (Figure 9).

Kinetics and Stoichiometry of PXP Formation. On the basis of spectral data, Martinez del Pozo et al. (*7*) found that D-alanine inactivation was accompanied by the disappearance of PLP absorbing at 415 nm and a corresponding increase around 335 nm. To verify whether this increase was caused by PMP or one of the proposed intermediates (*8*), we subjected inactivated samples to analysis for cofactor using the HPLC method after total denaturation. Inactivation caused a decrease in PLP and PMP peaks (Figure 10). In addition, a peak appeared around 8.8 min directly related to the inactivation, referred to as PXP, was found (Figure 8; lower panel). Furthermore, the PXP peak was closely connected to the percentage of inhibition (Figure 10).

Analysis of samples after reactivation (see above) showed that the reappearance of activity was accompanied by a disappearance of the PXP peak and a concomitant increase in PLP (Table 2), consistent with earlier observations that the spectrum of the reactivated enzyme had a peak around 415 nm (*7*). Based on the assumption that the total amount

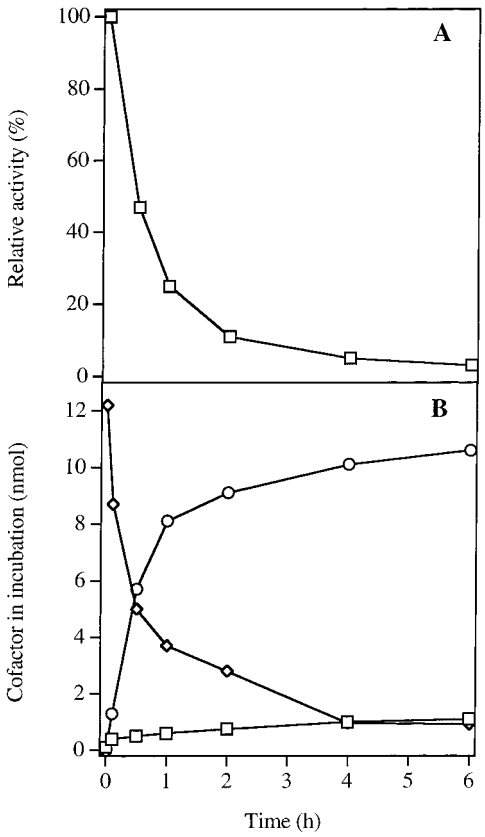


FIGURE 10: D-Alanine inhibition: relation between inactivation and cofactors. Enzyme (2.5 mg/mL) was incubated with 0.1 M D-alanine and 10 mM α-ketoglutarate. At the indicated time activity was measured (panel A) and after denaturation the enzyme cofactor was analyzed with the HPLC-based method (panel B): given are PLP (◇), PMP (□), and PXP (○).

Table 2: Cofactor Composition of Reactivated D-Amino Acid Transaminase[a]

time (h)	cofactor (nmol/mL incubation)		activity recovered (%)
	PLP	PXP	
2	0.05	2.4	6
20	0.45	1.5	35
92	2.0	0.22	70

[a] At the time points given activity was measured (as in Figure 7) and cofactor composition was determined. Given are the values found for the reactivation in 50 mM bis-Tris + 0.1 M KCl, pH 7.0.

of cofactor did not change during inactivation, a tentative correlation factor for PXP of 180 000 units/nmol was calculated (Figure 10). This value is in the range of those found for PMP and PLP (see above).

Protection by NADH and Correlation with PXP. We recently reported that the slow β-decarboxylation of D-aspartate leading to formation of D-alanine and inactivation could be prevented by the addition of NADH (*9*). As shown in Figures 11 and 12, this inactivation as well as its prevention by the reduced cofactor is closely correlated with the amount of PXP. NADH converted the cofactor into the PMP form, thereby confirming earlier spectral results (*9*). Addition of NADH after 2 h of D-aspartate-induced inactivation prevented further inhibition (*9*) and no additional PXP was formed, i.e., it even lowered the percentage of PXP somewhat, thereby converting it into PMP. NADH, however, was not able to protect the enzyme against direct

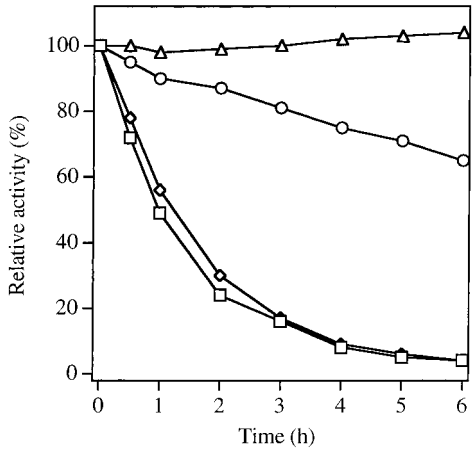


FIGURE 11: Effect of NADH on D-alanine and D-aspartate inhibition. Enzyme (2.0 mg/mL) was incubated with 0.1 M D-alanine or 1 mM D-aspartate in the presence or absence of 0.1 mM NADH: D-alanine − NADH (□); D-alanine + NADH (◇); D-aspartate − NADH (○), and D-aspartate + NADH (△). At the indicated time the activity was measured.

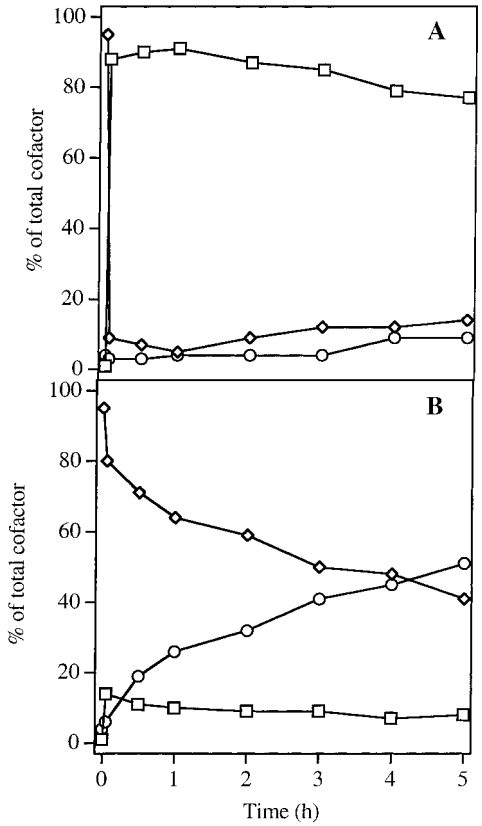


FIGURE 12: Cofactor composition of enzyme in the presence of D-aspartate. Enzyme (2.0 mg/mL) was incubated with 1 mM D-aspartate in the presence (panel A) or absence (panel B) of 0.1 mM NADH. Given are the percentages of the total cofactor content: PMP (□), PLP (◇), and PXP (○).

D-alanine inactivation (Figure 11), even when the concentration of the substrate was lowered to 1 mM. We ascribe this difference to the relative amounts of the E-PLP and the E-PMP forms of the enzyme in the presence of either D-aspartate or D-alanine. With D-aspartate there is only partial conversion of E-PLP to E-PMP and the effect of NADH is on the aldimine form of the enzyme. In contrast, with D-alanine most of the enzyme is converted to the E-PMP form and NADH is ineffective.

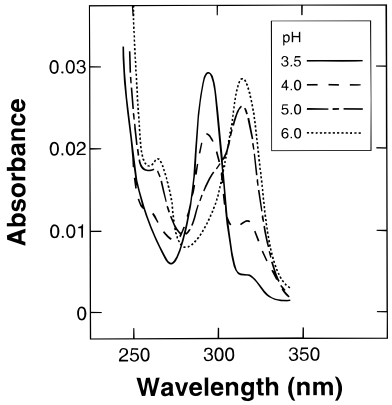


FIGURE 13: Spectra of PXP as a function of pH. Spectra of PXP (4.3 nmol) were recorded in 20 mM potassium phosphate in a final volume of 1.0 mL against water.

D-*Serine Inactivated Cofactor*. Analysis of D-serine-inactivated samples showed the appearance of a peak related to this inactivation in a similar relationship to that found for D-alanine. However, this peak eluted from the HPLC column at approximately 5.8 min, indicating that this intermediate is different from PXP but likely related to it, as would be expected from the presence of the 3′-OH group on the substrate.

Isolation of PXP. For large-scale isolation of PXP, 0.1% TFA (pH approximately 2.3) was used to avoid possible interference of the potassium phosphate in the mass spectrometric analysis. The elution volumes of the cofactors were only slightly affected by this change of eluent. With TFA, precautions must be taken to avoid a pH drop below 2, resulting in damage to the column material. By use of the described method, PXP could be isolated with a yield of 30−50% and at approximately 95−98% purity, based on the detected areas. The contamination was always PLP, which is produced from PXP upon storage (see below).

Stability of PXP. PXP is an unstable compound when kept at room temperature in 67 mM potassium phosphate, pH 2.3, or 0.1% TFA. Within 24 h it is converted to PLP. At 4 °C the stability improves, but after 1 week the isolated PXP peak disappears. However, PXP is stable at −20 or −80 °C (90−95% still present after 1 month). When kept as denatured enzyme, the decay in PXP is somewhat slower (5−10% remaining after 2 weeks). When stored as inactivated enzyme before denaturation, PXP is stable at room temperature for at least 2 days, at 4 °C at least several weeks, and at −20 or −80 °C. The instability at room temperature excludes the possibility that PXP is, in fact, PNP, which also elutes at a different position on the HPLC system used.

Spectral Properties of PXP. A PXP preparation containing approximately 5% PLP was used for recording spectra at different pHs (Figure 13). The initial spectra were recorded at the following pH values: 2.5, 4.5, 7.0, 9.5, and 11.5, assuming that the pK_as of the two phosphates, the phenol, the pyridinium, and a possible amine bound to the 4′ (or 4α) C (as is the case in PMP) are in the same range as those found in PLP and PMP (*14*). Spectra obtained for PXP were significantly different than for PLP and PMP (*15*). The results might indicate that the pK_a for the phenolic proton of PXP is higher than the corresponding groups in PLP and PMP, which is for both cofactors around 3.6. When spectra were recorded at pHs 3.5, 4.0, 5.0, and 6.0, the peak at 295 nm, still present at pH 3.5, gradually disappeared with increasing pH and a peak at 325 nm appeared, indicating that, indeed, the pK_a of the phenolic proton is higher than in PLP or PMP and is probably around 4.5. The differences in the spectra at pH 9.5 and 11.5 (not shown), which are similar to those found in PMP, indicate that it is likely that PXP, like PMP, contains an amine group at the 4′ (or 4α) carbon.

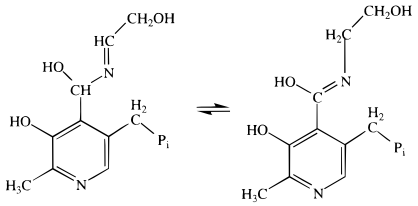


FIGURE 14: Proposed intermediate, PXP, for the inactivation of D-amino acid transaminase by D-alanine.

Mass Spectrometry. Analysis of purified PXP revealed a molecular mass of *m*/*z* 305 in the negative ion mode, while also a peak at *m*/*z* 246, indicating the decay product of PXP, namely, PLP, could be observed. Furthermore, a loss of *m*/*z* of 79 was also detected, indicating that PXP contained a phosphate group. All electrospray spectra of PXP under standard conditions (see Experimental Procedures) appear very weak; i.e., the intensities of sample-related peaks, even at the unusually high concentration used, are similar to background peaks. Presumably this is due to inherently low tendency of PXP either to lose protons or to be protonated. A set of consistently observed peaks, leading to a molecular mass of 306, can be ascribed to PXP. That is, $m/z = 307$ is observed in the positive ion mode and $m/z = 305$ in the negative ion mode and also in the phosphate loss mode. Altering the instrumental conditions to much gentler than normal, both the absolute intensity of the 305 peak and its value relative to the $m/z = 246$ PLP peak increased significantly. However, under these conditions peaks appear also at $m/z = 360$ and 419, with roughly the same intensity. From the collision-induced decomposition (CID) mass spectra, these are readily identified as TFA adducts of PLP and of the 305 peak. In the CID of the $m/z = 305$ ion, which becomes efficient at unusually low energies, the dominant pathway is the loss of neutral 59 to form $m/z = 246$, although some neutral loss of 18 is also seen. CID spectra of authentic PLP and the MS spectrum of the 246 ion formed from the fragmentation of the 305 ion are essentially identical and are consistent with what would be predicted for the PLP structure. The mass spectrometric observations indicate that the molecular mass 306 species is PXP. The data are consistent with the structure shown in Figure 14.

DISCUSSION

There are two major effects of salts described in this communication, i.e., inhibition of enzyme activity (Figure 3) and protection against D-alanine- or D-serine-induced inactivation (Table 1; *6*), that can be interpreted in the context of Scheme 1. The increased D-alanine-induced inactivation in the presence of decreasing concentrations of buffers (*8*) can be explained by decreased concentrations of chloride, the counterion in the buffers. Since salts impede the release of free pyruvate from the active site (Scheme 1, intermediate V) as shown by the results in Figure 4, the overall turnover

is decreased and the equilibrium of Scheme 1 would be shifted to the left, consistent with the spectral changes in the presence of D-serine as influenced by salts (Figure 7). Several lines of evidence support this proposal. First, in bis-Tris the pyruvate concentration increased quickly during the first minutes of incubation with D-alanine (Figure 4). However, titration experiments indicated that spectral changes from E-PLP to E-PMP were immediate (*11*). Therefore, the release of pyruvate from the enzyme (intermediate V) is a slow process compared to the coenzyme conversion to the PMP−ketimine complex (intermediate IV). Salt anions may stabilize intermediates I or II, thereby shifting the equilibrium in their favor.

The proposal that salts prevent the release of excess pyruvate from the enzyme is also supported by the results on the effect of salts on the coupled activity assay, i.e., increasing the salt concentration resulted in lower activities most likely due to decreased enzyme turnover (Figure 3). The reactivation experiments are also consistent with this proposal since in the presence of salts, PXP is transformed more quickly into PLP, indicating that in the absence of inhibiting substrates, salts protect or stabilize the PLP form of the enzyme (Scheme 1, intermediate II), perhaps by forming a complex with the protonated Schiff base to shift the overall equilibrium of Scheme 1 to the left. The effects of monovalent ions such as Na^+ and K^+ on pyridoxal enzymes have been reported (see ref *18* for a review on this subject), but the phenomena found in this study appear to be different, i.e., salts inhibit activity and formation of PXP by lowering the amounts of pyruvate produced rather than activating the enzyme (*18*).

The results with D-serine clarify the status of the various intermediates as well as the effect of salts on them because of its slower overall turnover relative to D-alanine. In Scheme 1 the conversion of E-PLP to E-PMP proceeds through quinonoid A (intermediate III), which is not usually detectable with good substrates but which is observed with poor substrates such as D-serine (*6*). Both in the absence as well as in the presence of salts there is an immediate decrease in the absorbance at 415 nm with a concomitant increase around 335 nm. The rates of these changes occurred too rapidly to measure on our instrument. In the absence of salts the subsequent appearance of the quinonoid absorbing at 492 nm occurs with D-serine at a slow rate (intermediate III and/or VI in Scheme 1). Quinonoid B (intermediate VI) is detectable at 492 nm with D-serine and is assumed to be the α-decarboxylated complex, while the third step is the conversion of quinonoid B into an inactivated cofactor PXP. Inactivation of the enzyme, which takes hours for completion, coincides with appearance of a compound corresponding to the PXP inactive form of the coenzyme derived from D-serine eluting at 5.8 min in the HPLC analysis (intermediate VII).

The extent of the increase at 415 nm and the concomitant decrease at 335 nm (Figure 7), which is more or less salt-independent, is likely due to a shift in the equilibrium between E-PLP and E-PMP. Extrapolating these results to D-alanine inactivation suggests that the D-alanine-derived quinonoid B, showing a small absorbance at 492 nm (*6*), is the decarboxylated compound in the scheme given by Bhatia et al. (*8*).

Since D-alanine is immediately converted into pyruvate, it can be argued that pyruvate or some form derived from it is required for this inactivation. However, free pyruvate added to the enzyme in the PLP or PMP form in the absence of D-alanine resulted in practically no inhibition. Pyruvate forms a nonproductive complex with the E-PLP form of the enzyme but could be expected to bind to the E-PMP form, although with a low affinity as described above. The finding that the D-alanine-induced inactivation increases with increasing enzyme concentration (Figure 1) can also be explained by Scheme 1, assuming that the complete system in equilibrium is a prerequisite. Hence, it is likely that inactivation is dependent on the concentration of pyruvate produced at the active site and that, at higher enzyme concentrations, more *free* pyruvate would be formed, thus driving the equilibrium in Scheme 1 to the left through quinonoid A to quinonoid B and PXP, thereby favoring inactivation.

It was found in previous studies using [^{14}C]-D-alanine that inactivation of one subunit of the dimeric enzyme renders the entire molecule inactive, based on the observation that the inactivated enzyme contained 1.1 mol of labeled cofactor adduct/mol of dimeric enzyme (*7*). This suggests that the remainder of the coenzyme is most likely still PLP or PMP. However, cofactor analysis using the HPLC-based method indicates that the percentage of PXP, likely to be that cofactor adduct, is about 85% after 6 h of incubation (Figure 10). This difference can possibly be ascribed to the fact that in the latter results the enzyme was inactivated using both D-alanine and α-ketoglutarate.

A structure of PXP is proposed that is consistent with the mass spectrometric results and earlier experiments (*7, 8*) in which it was found and confirmed here that, upon reactivation at acidic pHs, acetaldehyde and NH_4^+ were concomitantly formed with PLP, and inactivation probably is preceded by CO_2 release. This suggests that PXP is most likely an amine bound to a C_2 fragment attached to the 4′-C of the coenzyme. The small but significant differences observed when comparing spectra at pH 9.5 and 11.6 suggest that, indeed, the amine is present. The fact that the pK_a of the phenolic proton is shifted to a higher value compared to that in PLP or PMP might indicate that this proton is bound somewhat more tightly to PXP, possibly influenced by the presence of the extra group, i.e., the hydroxyl group on the 4′-C, which may form a hydrogen bond with that proton, thereby stabilizing it. Therefore, we suggest a structure of PXP as depicted in Figure 14, thereby defining the E_{inact} in the reaction scheme as proposed by Bhatia et al. (*8*).

The recent report that D-aspartate-induced inactivation is prevented by NADH (*9*) was found to correlate very well with a decrease in PXP formation (Figure 12). D-Alanine-induced inactivation is not prevented by NADH nor is there an effect on PXP formation by NADH. The major difference between these two D-amino acid substrates is that with D-aspartate there is only partial conversion of intermediate II to intermediate IV, whereas with D-alanine this conversion is nearly complete. Hence, NADH likely acts by donating a fragment (a hydride or a proton and an electron) to some intermediate, perhaps II, III, or VI, to form intermediate IV or V, neither of which can further process D-amino acids. It is unlikely that NADH reacts with PXP since E-PMP formed with NADH in the presence of D-aspartate is very rapid under conditions where there is little PXP.

The inactivation described here is affected by salts, while other physiological compounds, i.e., mono- and dicarboxylates and thiols, also show protective behavior (*8*). This could be an indication that in vivo regulation of the enzyme is determined by a complex of compounds, thereby ensuring adequate responses on the requirements of the cell.

ACKNOWLEDGMENT

The expert assistance of Maria A. Pospischil in the purification of the enzyme is gratefully acknowledged.

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BI972842P