The ubiquitous cofactor NADH protects against substrate-induced inhibition of a pyridoxal enzyme*

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Abstract

In the usual reaction catalyzed by D-amino acid transaminase, cleavage of the α -H bond is followed by the reversible transfer of the α -NH₂ to a keto acid cosubstrate in a two-step reaction mediated by the two vitamin B₆ forms pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP). We report here a reaction not on the main pathway, i.e., β -decarboxylation of D-aspartate to D-alanine, which occurs at 0.01% the rate of the major transaminase reaction. In this reaction, β -C-C bond cleavage of the single substrate D-aspartate occurs rather than the usual α -bond cleavage in the transaminase reaction. The D-alanine produced from D-aspartate slowly inhibits both transaminase and decarboxylase activities, but NADH or NADPH instantaneously prevent D-aspartate turnover and D-alanine formation, thereby protecting the enzyme against inhibition. NADH has no effect on the enzyme spectrum itself in the absence of substrates, but it acts on the enzyme-D-aspartate complex with an apparent dissociation constant of 16 μ M. Equivalent concentrations of NAD or thiols have no such effect. The suppression of β -decarboxylase activity by NADH occurs concomitant with a reduction in the 415-nm absorbance due to the PLP form of the enzyme and an increase at 330 nm due to the PMP form of the enzyme. α -Ketoglutarate reverses the spectral changes caused by NADH and regenerates the active PLP form of the enzyme from the PMP form with an equilibrium constant of 10 μ M. In addition to its known role in shuttling electrons in oxidation-reduction reactions, the niacin derivative NADH may also function by preventing aberrant damaging reactions for some enzyme-substrate intermediates. The D-aspartate-induced effect of NADH may indicate a slow transition between protein conformational states if the reaction catalyzed is also slow.

Keywords: D-amino acids; enzyme; enzyme inhibition; NADH; transaminase

NADH functions as an electron donor in many oxidation-reduction reactions. It is a loosely bound cofactor for several hundred enzymes that catalyze reactions ranging from lactate production to cholesterol biosynthesis, and is present at micromolar concentrations in many cell types (Dixon & Webb, 1979). Here we report a novel role for NADH, i.e., its effect on a pyridoxal 5'-phosphate enzyme-substrate complex. The enzyme in which this effect was found is D-amino acid transaminase, a potential target for the design of novel antimicrobial agents because it catalyzes the formation of D-alanine and D-glutamate, components of the peptidoglycan portion of the bacterial cell wall (Martinez-Carrion & Jenkins, 1965; Manning et al., 1974; Soper et al., 1977; Gale et al., 1981;

Martinez del Pozo, 1989a, 1989b; Futaki, 1990). The usual two-step reaction that this and other transaminases catalyze for amino acid and keto acid substrate pairs is shown in Equation 1:

These equations represent the reversible transfer of an amino group between amino acid (aa) and keto acid (ka) substrate pairs mediated by the two forms of the coenzyme, pyridoxal 5'-phosphate (PLP; E_L in above equation) and pyridoxamine 5'-phosphate (PMP; E_M in above equation). The reactions described in Equation 1 pertain to both D-amino acid transaminase, which accepts most D-amino acids as substrates (Jones et al., 1985), and L-aspartate transaminase, which prefers the dicarboxylic L-amino acids as substrates (Christen & Metzler, 1985). The E_L coenzyme form binds amino acid in an aldimine structure and the E_M form binds keto acid in a ketimine structure. Bond cleavage in transamination in-

*This work is dedicated to the memory of Dr. Alton Meister, whose many contributions to biochemistry continue to provide the foundations for current research.

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volves the α -carbon and its attached functional groups, i.e., the α -amine or α -ketone and the α -hydrogen atom of substrates. Processing at the β -carbon atoms is not on the major enzymic pathway, although β -elimination reactions with β -halo substrates have been reported with this enzyme (Manning et al., 1974; Soper et al., 1977) as well as for other transaminases (Christen & Metzler, 1985).

In order to appreciate the complete activity profile of this enzyme, we have been systematically studying the cleavage of other covalent bonds of individual substrates (Manning et al., 1974; Soper et al., 1977; Martinez del Pozo et al., 1989a, 1989b; Futaki et al., 1990), sometimes referred to as side reactions, of other pyridoxal enzymes (Manning et al., 1968; Braunstein, 1973; Soda & Tanizawa, 1979; Christen & Metzler, 1985; Miles, 1985). A finding that indicated the importance of these minor reaction pathways was that 3-amino-2,3-dihydrobenzoic acid (gabaculine), which is unrelated to normal substrates, inhibits the enzyme very efficiently by cleavage of a β -hydrogen atom rather than the usual α -hydrogen atom and thereby effectively inhibits bacterial growth (Soper & Manning, 1978). The reaction described in this communication also involves cleavage of a β -carbon bond when only the single substrate, D-aspartate, is present, i.e., the β -decarboxylation of its β -COOH groups to form D-alanine. This reaction is analogous to that described by Meister and colleagues, catalyzed by a specific aspartate β -decarboxylase (Meister et al., 1951; Nishimura et al., 1962; Novogrodsky et al., 1963), and by Almo et al. (1994). Graber et al. (1995) recently reported that wild-type L-aspartate transaminase catalyzed a very slow β -decarboxylation of L-aspartate to L-alanine. In the studies described here, we report an analogous reaction and also that the D-alanine produced from D-aspartate slowly inhibits the enzyme but NADH, which is not involved in PLP-catalyzed reactions, completely suppresses the β -decarboxylation and protects against enzyme inhibition.

Results

D-Aspartate is slowly decarboxylated to equivalent amounts of alanine by pure D-amino acid transaminase (Fig. 1) and eventually the reaction is complete (not shown); no other amino acids are detected by amino acid analysis. The alanine formed is the D-isomer,

Fig. 1. Correlation between D-aspartate turnover, D-alanine formation, and inhibition of transaminase activity. Experimental details are described in the text.

as determined by a procedure that we described recently (Jones et al., 1994). This result indicates that the alanine is produced at the active site of the enzyme. Possible decarboxylation of D-glutamate catalyzed by D-amino acid transaminase was studied by substituting D-glutamate for D-aspartate. The products of either α - or γ -decarboxylase activity on glutamate would be γ -aminobutyric acid or α -aminobutyric acid, respectively, but neither was found by amino acid analysis.

With an enzyme preparation purified to homogeneity (see Materials and methods), a specific activity of 0.025 μ moles of alanine $\text{min}^{-1} \text{mg}^{-1}$ of pure enzyme at 25°C (average of three experiments) $\pm 10\%$ was calculated. This rate of β -decarboxylation was compared with a rate of 200 μ moles $\text{min}^{-1} \text{mg}^{-1}$ of pyruvate in the normal transamination of D-alanine and α -ketoglutarate with the same enzyme preparation. Thus, the β -decarboxylase rate is approximately 0.01% the rate of transamination. The $V_{\max}$ pH profiles for β -decarboxylase activity had an optimum between pH 7.1 and 7.5, which was the same whether aspartate consumption or alanine formation was measured. The pH optimum for transamination for either wild-type or two active site mutant enzymes was between pH 8.1 and 8.5 (Yoshimura et al., 1992), suggesting a different mechanism for the two reactions consistent with the different sites of substrate bond cleavage.

Studies to test for impurities

That both reactions are catalyzed by a single enzyme was indicated by several types of controls: (1) both reactions were inhibited to a similar extent by three different inhibitors; (2) a recombinant enzyme had the same β -decarboxylase/transaminase ratio as the wild-type enzyme; and (3) removal or immobilization of the coenzyme affected both activities similarly. With respect to the effects of inhibitors, gabaculine, which is a very efficient inhibitor of normal transaminase activity catalyzed by D-amino acid transaminase (Soper & Manning, 1978), also completely inhibited the conversion of D-aspartate to D-alanine at a concentration of 6 μ M. Other previously reported inhibitors of the enzyme, i.e., D-serine (0.1 M) and methyl acetyl phosphate (1 mM) (Martinez del Pozo et al., 1989b), each completely inhibited both transaminase and decarboxylase activity. In a second test for possible impurities, a recombinant mutant enzyme with an altered activity toward normal substrates was also tested for β -decarboxylase activity; the purified recombinant enzyme K267A, in which the Lys side chain at the entrance to the active site was replaced by Ala (P.W. van Ophem & J.M. Manning, in prep.), had a reduced specific activity for both transaminase activity and β -decarboxylase activity. In addition, the native apoenzyme from which PLP had been removed by mild treatment with phenylhydrazine had less than 5% transaminase and decarboxylase activity remaining. Finally, the reduced enzyme, in which the internal aldimine of the coenzyme PLP linked to Lys-145 was reduced with NaCNBH_3 , possessed transaminase and β -decarboxylase activities that were decreased to about the same extent, i.e., 1–3%. Together these results strongly suggest that a trace impurity is not responsible for the β -decarboxylation of D-aspartate.

Inhibition of the enzyme and its protection

The conversion of D-aspartate to D-alanine was accompanied by inhibition of the enzyme, which was slower than alanine formation, presumably while the latter accumulated (Fig. 1); slow substrate-induced inhibition of this enzyme by D-alanine as the sole substrate has been reported previously and evidence provided

for a mechanism in which alanine is cleaved to acetaldehyde, ammonia, and CO_2 (by α -decarboxylation) (Martinez del Pozo et al., 1992; Bhatia et al., 1993). In the present investigation, attempts to detect either oxaloacetate from D-aspartate or pyruvate from D-alanine with malate dehydrogenase or lactate dehydrogenase, respectively, were negative. However, surprisingly, the turnover of aspartate to alanine was suppressed abruptly and completely. This protective effect was due entirely to the presence of NADH, the cofactor for these two dehydrogenases, because, when NADH was tested alone either at initial times or at 2 h after the beginning of the experiment at 30, 60, 100, and 500 μM , turnover of aspartate ceased immediately (Fig. 2, left panel) and alanine formation as well as enzyme inhibition were prevented (Fig. 2, right panel) (NADPH at the single 300 μM concentration tested was just as effective as NADH). In contrast, NAD did not reduce the conversion of D-aspartate to alanine, i.e., the time points for each in Figure 2 were identical, nor did it prevent inhibition of the enzyme; dithiothreitol, mercaptoethanol, or glutathione at equivalent concentrations were also without effect. In the presence of both co-substrates used to measure normal transaminase activity, there was no effect of NADH and a probable reason for this is discussed below. Hence, the protective effect of NADH is peculiar to the β -decarboxylation reaction pathway in the presence of a sustained concentration of D-aspartate alone.

Interpretation based on spectral changes

The results are interpreted according to Equation 2.

The major coenzyme absorption band corresponding to the PLP form of the wild-type enzyme (E_L), linked to Lys-145 in an internal aldimine structure in the absence of substrates, is at 415 nm (Fig. 3A). NADH has no effect on this absorption band (Fig. 3A). Binding of D-aspartate to the enzyme, which is known to be very fast (Jones et al., 1985), generates the external aldimine complex [E_L D-Asp], which also absorbs at 415 nm (Fig. 3B). Part of this complex is immediately converted to 330 nm due to formation of the PMP coenzyme. As D-alanine is slowly formed, the enzyme is also inhibited through a mechanism involving α -decarboxylation and formation of acetaldehyde (Martinez del Pozo et al., 1992; Bhatia et al., 1993). Addition of NADH results in an immediate further reduction in the 415-nm spectral band (Fig. 3C) and a corresponding increase in the 330-nm absorbance to an extent that is proportional to its concentration (see below). The experiment in Figure 3C was performed with an intermediate concentration of NADH (100 μM). These results were substantiated by using thio-NADH, which absorbs at 399 nm instead of 340 nm, the absorption maximum of NADH. The proximity of the latter absorption maximum to that of the PMP form (E_M) of the enzyme precluded quantitative identification of either of them. However, with thio-NADH, a distinct decrease was observed at 399 nm, dependent on the presence of D-aspartate, consistent with a reduction of the enzyme-D-aspartate complex by thio-NADH. This result indicates that the cofactor does not suppress decarboxylation by simply acting as a competitive inhibitor of substrate binding.

Transfer of hydride to the pyridoxal enzyme was tested with a commercial sample of tritiated pyridine nucleotide designated as radiolabeled with ^3H at C-4, the position involved in the redox events of the NADH system. However, we have no independent confirmation of structure of this compound. Using an HPLC system to separate the cofactors (P.W. van Ophem & J.M. Manning, unpubl.), PMP eluted at 3.8–4.0 mL, PLP at 7.0–7.5 mL, and NADH at 7.2–8.4 mL. When the $4\text{-}^3\text{H}$ cofactor was incubated with enzyme in the presence of D-aspartate but not in its absence, ^3H was found in the PMP region (6,000 cpm, representing 15–20% of the label in the eluted cofactor regions, a fraction of total label).

Enzyme Protection by NADH

Fig. 2. Left: Effect of NADH or NAD on decarboxylation of D-aspartate. NADH at a final concentration of 300 μM was added either initially or 2 h after the start of the incubation. Right: Effect of NADH on the formation of alanine and on enzyme transaminase activity. NADH at a final concentration of 300 μM was added at initial times. Enzyme transaminase activity was measured in the presence of α -ketoglutarate as described in the text. The decrease in transaminase activity at 2 h was proportional to the overall incubation time.

Fig. 3. Spectral changes and effect of NADH on the conversion of aspartate to alanine and effect of NADH. **A,B,C:** Initial enzyme concentration was 0.6 mg/mL. In B and C, initial D-aspartate concentration was 1 mM in 0.1 M bis-Tris, pH 7.4. In A and C, NADH concentration was 100 μ M; at this concentration, its large absorbance at 340 nm obscured the E-PMP (E_M) absorbance at 330 nm.

These results are consistent with hydride transfer to the pyridoxal cofactor attached to D-aspartate, although the mechanism by which it occurs requires further study.

The spectral changes on NADH addition (Fig. 3C) occurred concomitantly with cessation of aspartate turnover, alanine formation, and loss of activity (Fig. 2). These results indicate that an inactive complex is formed either by NADH itself or by some fragment of it. A possible analogous reaction takes place with the reducing agents NaCNBH_3 and leads to rapid reduction of the 415-nm absorbance band by donation of a hydride anion to the imine to give an inactive enzyme absorbing at 330 nm. Other effects of NADH on the enzyme are under study.

Binding efficiency of NADH

The K_m for D-aspartate in the usual transaminase assay is 0.4 mM (Bhatia et al., 1993). For the decarboxylase studies, a concentration of 1.0 mM D-aspartate was chosen; increasing its concentration to 10 mM gave a twofold increase in rate, which did not increase significantly with 50 mM D-Asp. The decarboxylase rate, which is slow and irreversible, provides an accurate measure of remaining active [E_L D-Asp] as a function of NADH concentration. A plot similar to that described by Mildvan and Leigh (1964) was used to obtain an apparent dissociation constant for the cofactor. The abscissa intercept (Fig. 4) corresponds to an apparent K_d of 16 μ M, which is close to the concentration of enzyme active sites used (22 μ M), indicating that the NADH effect is highly efficient.

Effect of α -ketoglutarate

Increasing concentrations of α -ketoglutarate result in a proportional shift of the spectrum of the NADH-suppressed enzyme from 330 to 415 nm (Fig. 5A), which is identical to the conversion of the E_M form to the E_L form of the enzyme. The efficiency of the α -ketoglutarate effect is illustrated by the analysis of the titra-

tion curve at 415 and 330 nm (Fig. 5B). The concentrations of α -ketoglutarate needed for 50% conversion between the E_L and E_M coenzyme forms are 11 μ M and 9 μ M for these two wavelengths, respectively. This value is approximately equivalent to the enzyme concentration used. Thus, the efficiency with which α -ketoglutarate regenerates the E_L form of the enzyme is close to the 16 μ M concentration at which NADH binds to the enzyme to exert its suppressive effect (Fig. 4).

α -Ketoglutarate also resulted in an enhanced β -decarboxylation rate because both aspartate turnover (Fig. 6A) and alanine formation (Fig. 6B) were increased in its presence. These results are consistent with the α -ketoglutarate titration results in Figure 5A and B, and indicate that it is the E_L form of the enzyme that is active in catalyzing β -decarboxylation. These data suggest that the form of the enzyme present after the rapid decrease of the 415-nm absorbance (Fig. 3C) by NADH is the E_M form of the enzyme, which cannot form a productive complex with D-aspartate. Because Equation 2 indicates that β -decarboxylation proceeds from the E_L form of the enzyme, conversion to the E_M form would therefore suppress this reaction by a mechanism whereby NADH converts the $\text{CH}=\text{NH}$ —azomethine linkage to a $\text{CH}_2-\text{N}=\text{imine}$ linkage, or to E_M , neither of which would be reactive with D-aspartate, as shown by Meister and his colleagues (1951), but which could be regenerated to E_L by α -ketoglutarate. These results (Figs. 5, 6) also explain why NADH has no effect on transaminase activity measured in the presence of α -keto acids, which regenerate the E_L form of the enzyme even if reduced by NADH.

Discussion

Several groups (Kirkman & Gaetani, 1984; Jouve et al., 1989; Hillar & Nicholls, 1992) have demonstrated that catalase from both eukaryotic and prokaryotic sources contains bound NADPH, which, although not involved in the catalytic function of this heme-containing enzyme, protects the enzyme against free radical damage in the presence of the substrate H_2O_2 . The studies reported

Fig. 4. Apparent dissociation constant of NADH binding to enzyme D-aspartate complex. Experimental conditions were identical to those described for Figure 3 except that the concentration of NADH was varied from 10 to 40 μ M and the amount of decarboxylase activity remaining was measured by the rate of conversion of D-aspartate (1 mM) to D-alanine. Rates were linear over a period of several hours, consistent with the irreversible nature of the β -decarboxylation. Enzyme concentration was 11 μ M in dimer or 22 μ M in active sites.

Fig. 5. A: Effect of α -ketoglutarate on the spectrum of the enzyme after NADH treatment. Incubation of enzyme (10 μ M) with NADH (100 μ M) and D-Asp (1 mM) was performed in 0.1 M bis-Tris, pH 7.4, to give the maximal conversion of 415 nm to 330 nm. In order to remove excess NADH and D-Asp, the incubation mixture was transferred to an Amicon Centricon 10 device and centrifuged at 6,000 rpm to approximately 0.1 mL. Three washings with 1.0 mL of the same buffer were performed and the sample was again centrifuged to $\sim$ 0.1 mL each time. After the last centrifugation, 0.9 mL of the same buffer was added to the $\sim$ 0.1 mL enzyme solution remaining. The spectrum from 300 to 520 nm was recorded on a Cary 2200 before and after adding 3–10- μ L amounts of concentrated α -ketoglutarate to give the concentration shown in the figure. Spectral changes shown occurred immediately and did not change with time. B: Extent of the total spectral decrease at 330 nm was set at 1.0 and the fractional decrease after each addition of α -ketoglutarate was plotted. For the 415-nm changes, the fractional increases were subtracted from 1.0, which was taken as the total spectral increase at this wavelength.

Fig. 6. Enhancement in the rate of β -decarboxylation by α -ketoglutarate. Experimental details are described in the text. When present, α -ketoglutarate was at concentrations of 0.2 mM or 0.5 mM, with the same results at each concentration. A: Aspartate turnover. B: Alanine formation.

here indicate a protective role by NADH similar to that found for catalase, even though the details of the process may be different. The recently solved structure of native D-amino acid transaminase (Sugio et al., 1995) does not reveal the presence of bound NADH, but does not exclude a binding site for it. Even though free radical chemistry is not generally involved in pyridoxal-mediated catalysis, NADH affects the C-4 of the coenzyme-aspartate complex (Equation 2, bold type) to generate the E_M form, perhaps by transfer from a distance through the protein matrix, i.e., tunneling (Huber, 1990; Gray & Winkler, 1996). The C-4 of the coenzyme is the site of protonation that actively participates in normal transamination (Yoshimura et al., 1996). The nature of the enzyme side chain involved in proton transfer to the coenzyme has been a subject of considerable interest, but its identity has remained elusive.

The results described here have several ramifications. One is a general NADH involvement in protecting enzymes in which it is not involved in catalysis. The precursor of the nicotinamide ring of NADH is the vitamin niacin, but this widely distributed cofactor has not been considered previously to be an antioxidant (reductant). Finally, the susceptibility of the bound coenzyme to the effects of NADH in the presence of D-aspartate but not in its absence, even though coenzyme-aldimine linkages are present in either instance, is reminiscent of the Koshland induced-fit model (Yankeelov & Koshland, 1965). Transitions between some protein conformational states may be slow when the reaction catalyzed is also slow.

Methods and materials

Measurement of transaminase and decarboxylase activity

Bacterial D-amino acid transaminase (wild-type) was purified as described previously and an additional gel filtration step on FPLC using Superose-12 was introduced. The pure enzyme, which displayed one band by SDS-PAGE chromatography, had a specific activity of 200 units/mg at room temperature for transaminase activity using the lactate dehydrogenase-coupled transaminase assay. Transaminase activity was measured with the substrates D-alanine (200 mM final concentration) and α -ketoglutarate (25 mM final concentration) in 0.1 M EPPS, pH 8.1, at 25 °C (P.W. van Ophem & J.M. Manning, unpubl.). To 970 μ L of this mixture was added 10 μ L of wild-type enzyme containing 1–10 μ g of protein and 10 μ L of a 1:10 diluted solution of lactate dehydrogenase containing 200 μ M NADH in a 1-mL cuvette in a Cary 2200 spectrophotometer. The rate of transamination was calculated as the slope of the decrease in absorbance at 340 nm as a function of time.

To measure β -decarboxylation, the enzyme (300 μ g total, 0.65 mg/mL, 11 μ M in dimer, 22 μ M in active sites) was incubated with 1 mM D-aspartate at 37 °C in 0.1 M bis Tris, pH 7.4. At the indicated times, 90- μ L aliquots were removed and added to 10 μ L of cold 50% sulfosalicylic acid. After centrifugation, 40 μ L of the supernatant were added to 60 μ L of 0.2 M Na citrate, pH 2.2, and the final pH adjusted to 2.2 ± 0.2 with NaOH. The amounts of aspartate and alanine were determined on a Beckman 6300 amino acid analyzer with a System Gold data handling system. NADH, NADPH, and thio-NADH were purchased from Sigma and added at the concentrations and times indicated.

Calculation of the apparent dissociation constant for NADH

The remaining decarboxylase activity as a function of added NADH concentration was measured as the amount of aspartate turnover

and alanine formation per unit time. Rates of decarboxylation were determined by measuring the aspartate to alanine conversion for at least six determinations over a period of 2–3 h; the rates were linear. Because decarboxylation is irreversible and inactivation of a certain percentage of enzyme molecules was directly related to NADH concentration, the data were treated as described by Mildvan and Leigh (1964) to obtain the apparent dissociation constant for NADH.

Calculation of the equilibrium constant for α -ketoglutarate

The effect of increasing concentrations of α -ketoglutarate in shifting the absorption spectrum of the NADH-treated enzyme from 330 to 415 nm was evaluated. After treatment of the enzyme with D-aspartate and NADH, and their subsequent removal as described in the legend to Figure 5, the fractional changes at either 415 or 330 nm were plotted as a function of α -ketoglutarate concentrations on a logarithmic scale. The apparent binding constant was calculated as that concentration of α -ketoglutarate that resulted in a 50% change in the 330-nm absorbance or the 415-nm absorbance, where the concentrations of each component are equal.

Studies with isotopic 4- 3 H pyridine nucleotide cofactor

This compound was obtained from Amersham and mixed with unlabeled NADH (final conc.: 100 μ M) and enzyme in the presence of D-aspartate (1 mM) or its absence. After incubation, a portion was applied to an HPLC column to separate the cofactors (P.W. van Ophem & J.M. Manning, unpubl.) as described in the text.

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