Mechanism-Based Inactivation of L-Aspartase from *Escherichia coli*†

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ABSTRACT: The substrate analogue L-aspartate β-semialdehyde (L-ASA) has been identified as a mechanism-based inactivator of L-aspartase from *Escherichia coli*. The enzyme catalyzes the deamination of L-ASA to yield fumaric acid semialdehyde (FAA) and NH_4^+, with the product FAA partitioning between subsequent release or irreversible enzyme inactivation. Complete protection against L-ASA inactivation is observed in the presence of the product fumarate and a divalent metal ion. However, protection against inactivation by the product FAA also requires the presence of an enzyme activator. In addition to functioning as a mechanism-based inactivator, L-ASA has also been shown to serve as an activator of L-aspartase. The mechanism of inactivation by FAA involves the attack of an active site nucleophilic at the α-carbon of FAA to yield a stable Michael type enzyme adduct. Subsequent formation of a hydrazone upon treatment of the enzyme adduct with 2,4-dinitrophenylhydrazine confirms the presence of the unreacted aldehydic group of FAA. Examination of a group of product analogues with different substituents has demonstrated a correlation between the electron-withdrawing ability of these functional groups and the rate of inactivation of L-aspartase.

The enzyme L-aspartase catalyzes the reversible deamination of L-aspartic acid to yield fumarate and ammonium ion. The enzyme from *Escherichia coli* is composed of four identical subunits, with an overall MW of 208 kDa (Takagi et al., 1985). L-Aspartase is a textbook example of a highly specific enzyme (Lehninger, 1982), with no alternative substrates having been identified (Falzone et al., 1988). The enzyme requires a divalent metal ion for catalytic activity at pH 7.5 and above (Suzuki et al., 1973), and a lag time is observed in the amination direction in this pH range (Ida & Tokushige, 1985b; Karsten et al., 1986). This reaction lag time can be reduced, or eliminated, by the addition of L-aspartic acid or by certain structural analogues. These analogues have been shown to be noninhibitors of the enzyme, which has led to the hypothesis that L-aspartase is composed of two distinct sites, an activator site and an active site (Karsten et al., 1986). Chemical modification and pH profile studies have suggested the potential importance of cysteine (Mizuta & Tokushige, 1975), histidine (Ida & Tokushige, 1984; Karsten & Viola, 1991), and lysine residues (Karsten & Viola, 1991) in the activity of L-aspartase. Recent site-directed mutagenesis studies have identified a specific lysyl residue that appears to be involved in the active site of the enzyme (Saribas et al., 1994).

The substrate analogue L-aspartate β-semialdehyde (L-ASA)[1] has been reported to irreversibly inactivate L-aspartase (Yumoto et al., 1982), and preliminary work suggested that this compound was acting as a mechanism-based inactivator (Higashi et al., 1988). Since this earlier report, there has been no further characterization of the mechanism of L-ASA inactivation of L-aspartase.

We have examined the mode of action of the substrate analogue, L-ASA, and the product analogue, fumaric acid semialdehyde (FAA), to identify useful probes for the active site of L-aspartase. From these studies we present evidence that L-ASA is acting as a mechanism-based inactivator. Protection studies against L-ASA and FAA have aided in the identification of the mechanism of inactivation and have also provided evidence for cooperativity between the active and activator site of L-aspartase.

MATERIALS AND METHODS

Materials. L-Aspartic acid, fumarate, α-methyl-DL-aspartate (AMA), and L-allylglycine were all obtained from Sigma and were used without further purification. L-ASA was prepared by the ozonolysis of l-allylglycine and was purified according to a published method (Black & Wright, 1955). FAA was prepared by refluxing 20 g of methyl crotonate in 100 mL of dioxane for 4 h with 13 g of SeO_2 (Bohlman & Inhoffen, 1956). Treatment with this reagent leads to the selective oxidation of the allylic carbon to the aldehyde (Trachtenberg, 1969). Glutamate dehydrogenase (GDH), ammonia-free in glycerol was from Boehringer, and 2,4-dintrophenylhydrazine (DNPH) was from Aldrich. The enzyme L-aspartase was purified by the procedure of Karsten et al. (1985).

Enzyme Assay. The activity of L-aspartase was determined spectrophotometrically by measuring the formation of fumarate at 240 nm (E_{240} = 2.53 mM^{-1} cm^{-1}). A standard assay mix contained 30 mM L-aspartate, 5 mM $MgCl_2$, and 50 mM TAPS buffer, pH 8.0. To measure the lag time in the L-aspartase activity, the enzyme was assayed in the direction of aspartate production. The reaction conditions used were as follows: 50 mM TAPS buffer, pH 8.0, with 10 mM fumarate, 20 mM NH_4^+, and 5 mM Mg^{2+}, in the presence or absence of L-ASA.

Inactivation of L-Aspartase. L-Aspartase (20–35 μg) was incubated in a solution containing 400 mM of various sulfonic acid buffers (Good et al., 1966), depending on the pH, and from 0.1 to 3.5 mM L-ASA or from 1.0 to 5.5 mM FAA as the inactivator. Other inactivators were also examined by varying their concentrations in an appropriate range to observe enzyme inactivation. At given time intervals enzyme activity

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[1] Abbreviations: AMA, α-methylaspartic acid; L-ASA, L-aspartate β-semialdehyde; DNPH, 2,4-dinitrophenylhydrazine; FAA, fumaric acid semialdehyde; GDH, glutamate dehydrogenase.

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was determined by removing an aliquot (1–3 μL) from the incubation mix and adding it to 1 mL of the standard assay mix.

The kinetic parameters for L-ASA inactivation were found by using the method of Waley (1985). Equation 1 has been proposed to examine mechanism-based inactivators by using steady-state analysis:

$$I_o t_{1/2} = [\ln(2 - M)/(1 - M)](K_i/k_{inact}) + \ln 2/k_{inact}(I_o) \quad (1)$$

In this equation, $M = (1 + r)E_o/I_o$, where r is the partitioning ratio between catalytic turnover and enzyme inactivation. If the value of E_o/I_o is less than 1, then eq 1 reduces to

$$t_{1/2} = \ln 2(K_i/k_{inact})(1/I_o) + \ln 2/k_{inact} \quad (2)$$

Since eq 2 represents a first-order process, then $t_{1/2} = \ln 2/k_{obs}$. Substitution and rearrangement gives

$$1/k_{obs} = 1/k_{inact} + K_i/k_{inact}(1/I_o) \quad (3)$$

These inactivation studies were carried out by using an E_o/I_o ratio of 10^{-7} and were analyzed by plotting $1/k_{obs}$ *vs* $1/I_o$. The values of k_{inact} and K_i were then obtained by using a least-squares fit to the data.

pH Dependence of L-Aspartase Inactivation. The rate of inactivation (k_{inact}) of L-aspartase was examined at various pH values when incubated in the presence of 1.0 mM L-ASA. The data were fitted to eq 4 to obtain a pK_a value for the group that is involved

$$k_{inact} = \frac{k_{min} + k_{max}(K_a/[H^+])}{1 + (K_a/[H^+])} \quad (4)$$

where k_{min} and k_{max} are the minimum and maximum values of the rate of inactivation, respectively.

Determination of Partition Ratio. The partition ratio between product formation and enzyme inactivation for the mechanism-based inactivation of L-aspartase was found by using a titration method (Silverman, 1988). L-Aspartase was incubated with concentrations of L-ASA ranging from 5.0 to 180 μM. When no further loss of activity was observed, the remaining enzyme activity was then determined by using the standard assay mixture as described above. The percent activity remaining was plotted against the ratio of the concentration of L-ASA to the initial concentration of the enzyme. The partition ratio was then calculated from the slope of this line.

Ammonia Assay. A standard assay mixture contained 10 mM α-ketoglutarate, 0.1 mM NADPH, and GDH. A standard curve was constructed by adding known amounts of NH_4^+ to the assay mix and then determining the rate at 340 nm (Levitzki, 1970). L-ASA was then incubated with L-aspartase and, at given time intervals, aliquots were removed and added to the standard assay mix. The final determination of NH_4^+ concentration was made by using this standard curve after subtracting any background rate observed in the absence of L-aspartase. The low levels of ammonium ion that were produced ensured that GDH was operating in the first-order range.

Treatment of Modified L-Aspartase with DNPH. The carbonyl content of the protein was determined by using a variation of a previously described technique (Levine et al., 1990). The L-ASA-modified protein (0.5 mg) was centrifuged in an Amicon filter (MW cutoff = 30 000) and then was washed three times with buffer. The protein was collected and taken up in 6 M guanidine–HCl, pH 7.0. To this solution was added 250 μL of DNPH (20 mM stock in 2 N HCl). The reaction was allowed to stand at room temperature for 2 h. The samples were freeze dried to remove water and then washed with ethanol:ethyl acetate (1:1) until all of the unreacted DNPH was removed. The samples were then centrifuged, and the solvent was removed. The protein was redissolved in 6 M guanidine–HCl, pH 7.0, and the absorbance was measured at 370 nm, using E_{370} = 22 000 M^{-1} for the formation of the hydrazone (Johnson, 1953).

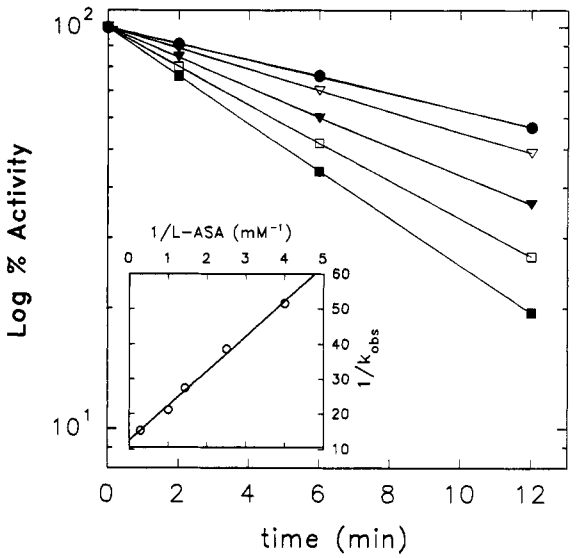

FIGURE 1: Time course for the inactivation of L-aspartase at pH 8.0. L-Aspartase (0.5 μM) was incubated at 25 °C with varying amounts of L-ASA. [L-ASA] = 0.25 mM (●); 0.40 mM (▽); 0.70 mM (▼); 1.00 mM (□); and 2.29 mM (■). The remaining enzyme activity was determined as described in the Materials and Methods. Inset: reciprocal plot of the observed rate constant (k_{obs}) for L-ASA inactivation of L-aspartase as a function of L-ASA concentration. The k_{obs} was determined for each concentration of L-ASA by calculating the $t_{1/2}$ for the reaction.

RESULTS

Inactivation of L-Aspartase. When L-aspartase is incubated in the presence of the substrate analogue, L-ASA, a time dependent loss of catalytic activity is observed (Figure 1). This inactivation follows saturation kinetics, suggesting that L-ASA is capable of forming a reversible complex with the enzyme before any covalent modification occurs (Silverman, 1988). A reciprocal plot of the first-order rate constant (k_{obs}) as a function of inactivator concentration is linear (Figure 1, inset), and the inactivation constant, K_i, and the rate constant for inactivation, k_{inact}, were determined from a fit to eq 3 to be 0.71 ± 0.08 mM and 0.082 ± 0.003 min^{-1}, respectively.

A time dependent loss of activity is also observed when L-aspartase is incubated in the presence of the product analogue, fumaric acid semialdehyde (FAA). A plot of the rate constant against the concentration of FAA gives a second-order rate constant for inactivation of 0.016 mM^{-1} min^{-1}.

Protection against L-ASA Inactivation. To identify the nature of the interaction of L-ASA with the enzyme, protection studies were carried out by the addition of products and activators to the incubation mixture. L-Aspartic acid was not used as a protectant since it has been shown to bind at both the active and the activator sites of the enzyme (Karsten et al., 1986). Different combinations of protectants were examined; however, only the presence of both fumarate and Mg^{2+} was found to provide complete protection against L-ASA

Table 1: Protection of L-Aspartase against Inactivation[a]

protectant[b]	% protection[c] ASA inactivation	FAA inactivation
Mg^{2+}	0	0
fumarate + EDTA	0	0
fumarate + Mg^{2+}	86	13
fumarate + EDTA + AMA	0	0
fumarate + Mg^{2+} + AMA	89	90
fumarate + Mg^{2+} + L-ASA	n.d.	95
AMA + EDTA	0	n.d.
AMA + Mg^{2+}	17	−55

[a] 20 μM L-aspartase was incubated in the presence of 2 mM L-ASA or FAA at pH 8.0 (400 mM TAPS). Aliquots were removed from the reaction mixture at various time intervals and assayed for enzyme activity. The ionic strength was kept constant by the addition of various amounts of KCl and had no effect on the rate of inactivation. [b] When present, compounds were added at the following concentrations: 10 mM fumarate, 20 mM AMA, 5 mM EDTA, 10 mM $MgCl_2$. [c] % protection = [1 − [(activity of control − activity with protectant)/(activity of control − activity without protectant)]]100.

inactivation (Table 1). These results indicate that L-ASA is capable of interacting at the active site of L-aspartase, since it has been shown that fumarate only binds at the enzyme active site (Falzone et al., 1988). From this study it is also clear that Mg^{2+} must be present in order for fumarate to protect against L-ASA inactivation. Binding studies have shown that in order for fumarate to bind to L-aspartase at higher pH (pH > 7.5), both a metal ion and an activator must be present (P. Vicedomine and R. E. Viola, unpublished results). This requirement for binding of the normal substrates suggests that, in addition to active site directed binding and inactivation, L-ASA can also bind at the activator site and enable fumarate to bind, if Mg^{2+} is present. To examine this possibility, conditions for the amination reaction were adjusted to result in a measureable reaction lag time. As had previously been observed, if L-ASA is an activator for the enzyme, then the binding of L-ASA at the enzyme activator site will reduce this lag time (Karsten et al., 1986). Addition of 1 mM L-ASA to this reaction mixture caused a decrease from 6 to 1 min in the lag time required to achieve a steady-state rate, consistent with L-ASA binding at the activator site.

Protection against FAA Inactivation. The results of the protection studies against L-ASA inactivation suggest a cooperative mode of binding of fumarate and activator. This model was further supported by protection studies against FAA inactivation (Table 1). Complete protection was observed in the presence of fumarate, Mg^{2+}, and AMA. Once again, no protection was observed with fumarate in the absence of either metal ion or activator, showing the need for both an activator and a metal ion in order for fumarate to bind. When AMA was replaced with L-ASA, complete protection was also observed (in the presence of Mg^{2+}), confirming the activator role of L-ASA and the inability of the product analogue FAA to play this role. The presence of AMA and Mg^{2+} alone offered no protection; in fact, the rate of inactivation of L-aspartase by FAA was found to increase under these conditions.

Evidence for Mechanism-Based Inactivation. It has been proposed that L-aspartase may catalyze the deamination of L-ASA to give FAA and NH_4^+ (Yumoto et al., 1982). While FAA has also been shown to inactivate the enzyme (Higashi et al., 1988, and this work), there has been no evidence reported to show that L-aspartase can catalyze the conversion of L-ASA to FAA. The production of either FAA or NH_4^+ upon addition of the enzyme to L-ASA would have to be demonstrated in order to confirm this hypothesis. It would be difficult to quantitate the amount of highly reactive FAA that is formed, so conditions were examined to determine the formation of NH_4^+. L-Aspartase (1.5 μM) was incubated with 4.2 mM L-ASA, pH 8.0, for periods of up to 12 h, at which time no enzyme activity remained. When aliquots of this incubation mix were removed at various times and assayed for NH_4^+ (see Materials and Methods), a time course for the production of ammonium ion was observed, which saturated at a final concentration of 370 μM NH_4^+ produced at the longest times. No production of NH_4^+ was observed when L-ASA was incubated under the same conditions and time periods in the absence of L-aspartase. An analogous reaction with L-ASA to that proposed for the physiological substrate would account for the production of NH_4^+. This mechanism features proton removal at carbon-3 to generate a carbanion, with subsequent elimination of NH_4^+ (Porter & Bright, 1980). The catalytic production of ammonium ion confirms that L-ASA is acting as a mechanism-based inactivator. Further, the inactivating species, FAA, can partition between convalent attachment or dissociation. This partition ratio was determined by the titration method as outlined in the Materials and Methods. Figure 2 shows a plot of E_o/I_o *vs* enzyme activity at pH 8.0. Extrapolation of this graph to 0% enzyme activity gives a partitioning ratio (k_{cat}/k_{inact}) of 29. This analysis also allows a calculation of the k_{cat} for L-ASA, which is 2.45 min^{-1}, compared to a k_{cat} of 6.6×10^2 min^{-1} for L-aspartic acid under these conditions.

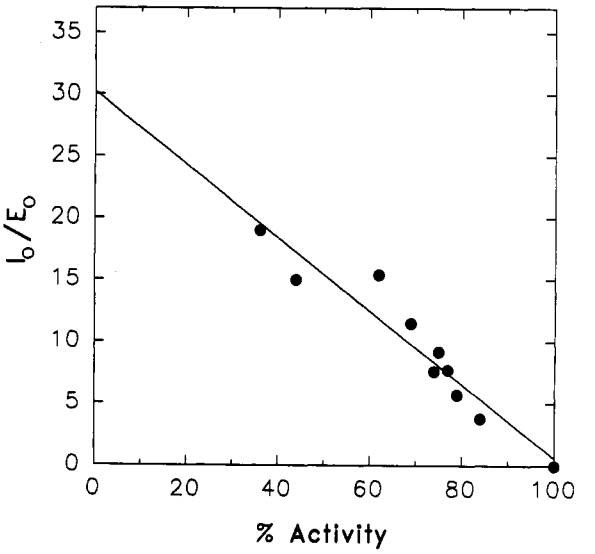

FIGURE 2: Plot of I_o/E_o *vs* the enzyme activity remaining at pH 8.0. L-Aspartase (0.7 μM) was incubated with different concentrations of L-ASA for 0–21 h. The excess L-ASA was removed, and activity was measured as described in the Materials and Methods.

Mechanism of L-ASA Inactivation. There are two reasonable adducts that could potentially form during the interaction of L-aspartase with FAA. The first is a Schiff's base between an active site lysine and the aldehydic group of FAA. Alternatively, nucleophilic attack at carbon-2 of FAA, due to the electropositive nature of the double bond, could also lead to a covalently modified enzyme. The type of adduct that forms when L-aspartase is incubated with L-ASA was investigated by treating the L-aspartase-ASA reaction mixture with DNPH. If a Schiff base is responsible for the adduct (with preservation of the double bond of FAA), then DNPH would react with this adduct to form a dissociable hydrazone and the free enzyme (Figure 3). However, if the aldehydic group is not the site of adduct formation, then formation of a nondissociable hydrazone adduct would be observed upon

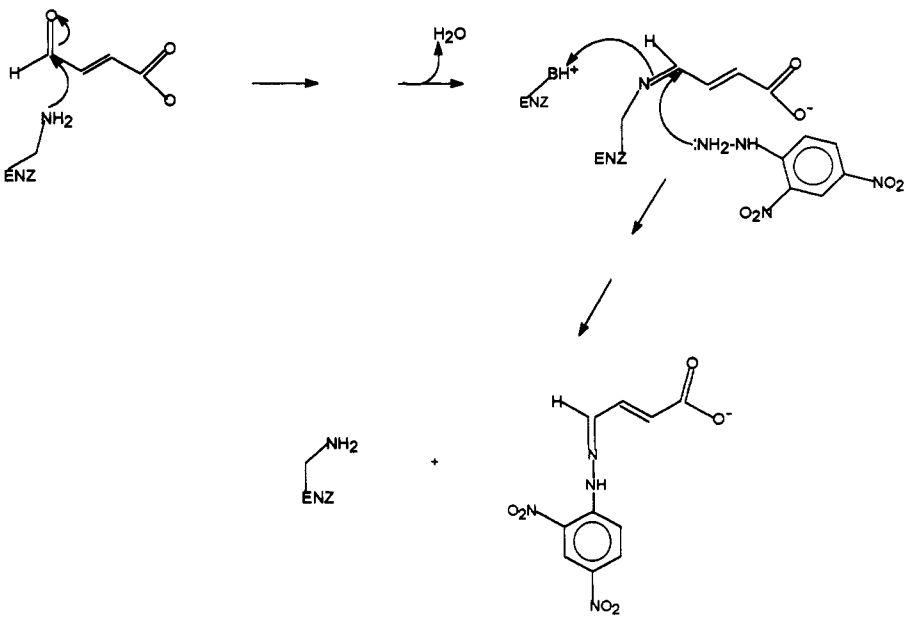

FIGURE 3: Proposed reaction of an active site lysine with the aldehydic group of FAA to form a Schiff base adduct. Subsequent derivatization with DNPH would yield the free enzyme and the hydrazone adduct.

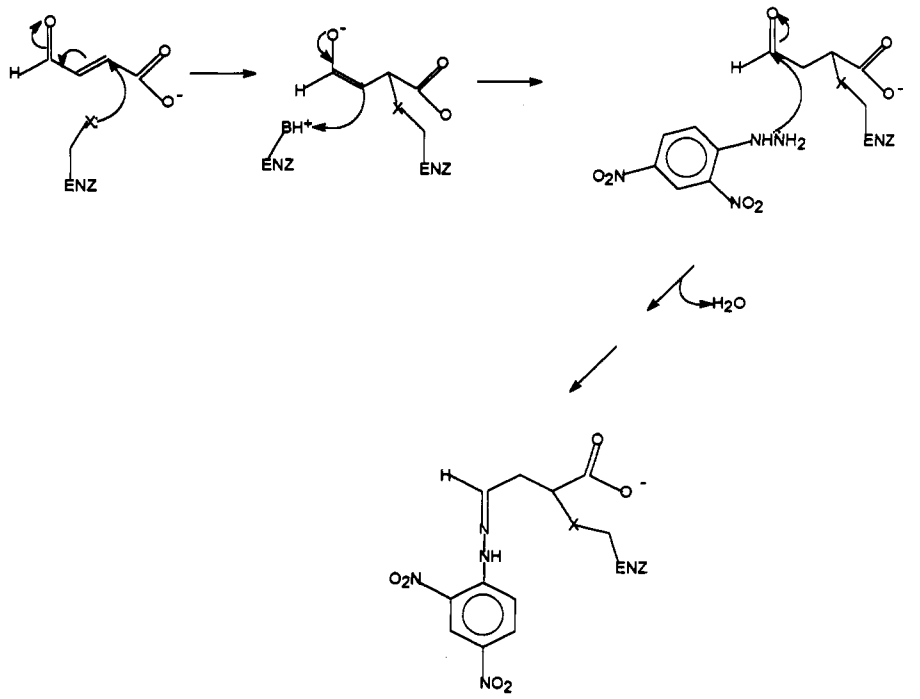

FIGURE 4: Proposed reaction of an active site nucleophile (X) with the double bond of FAA. Subsequent derivatization with DNPH would result in an enzyme-bound hydrazone adduct.

derivatization with DNPH (Figure 4). The ASA-inactivated enzyme was denatured by incubation in guanidine-HCl, followed by addition of DNPH. After treatment to remove the excess reagent, an increase in absorbance at 370 nm was observed, which saturated at a stoichiometry of 1. When L-aspartase was incubated with DNPH in the absence of L-ASA no change in absorbance was observed. These results suggest that the adduct formation between FAA and L-aspartase is due to attack of an enzyme active site nucleophile at carbon-2 of FAA (Figure 4).

A set of fumarate analogues were then examined for their ability to inactivate L-aspartase. Reaction according to the mechanism proposed in Figure 4 would be predicted to lead to an increase in the rate of enzyme inactivation as the site of nucleophilic attack is made more electropositive. This hypothesis was examined by the experiments reported in Table 2. The most potent analogue, β-nitroacrylate methyl ester, causes the inactivation of L-aspartase at a rate which is 10 orders of magnitude greater than that observed with FAA.

To identify the nature of the active site nucleophile that is responsible for adduct formation, the rate of inactivation was examined as a function of pH. At low pH no inactivation of L-aspartase is observed in the presence of L-ASA. As the pH increases enzyme inactivation is observed, increasing to a maximum rate at pH values greater than 10 (Figure 5). The rate of inactivation for FAA shows the same pH dependence as that of L-ASA; i.e., no inactivation is observed at pH 6.5 or below, and an increasing rate of inactivation is seen at higher pH values. The direction of this titration curve indicates that the ionized form of a group on the enzyme is required

Table 2: Inactivation of L-Aspartase by Substrate or Product Analogues[a]

analogue	R_1[b]	R_2[b]	K_{inact} (mM^{-1} min^{-1})
L-ASA	CHO	COOH	0.104
crotonaldehyde	CHO	CH_3	0.0071
FAA	CHO	COOH	0.016
β-nitroacrylate	NO_2	COOH	4.7×10^2
FAA α-methyl ester	CHO	$COOCH_3$	8.9×10^2
β-nitroacrylate methyl ester	NO_2	$COOCH_3$	3.6×10^8

[a] Reaction conditions were as follows: 400 mM HEPES, pH 8.0, and varying concentrations of inactivators. Aliquots were removed from the reaction mixture at various time intervals and assayed for enzyme activity. [b] Parent compound: $R_1CH{=}CHR_2$, except for the substrate analogue, L-ASA, which has ammonium ion added across the double bond.

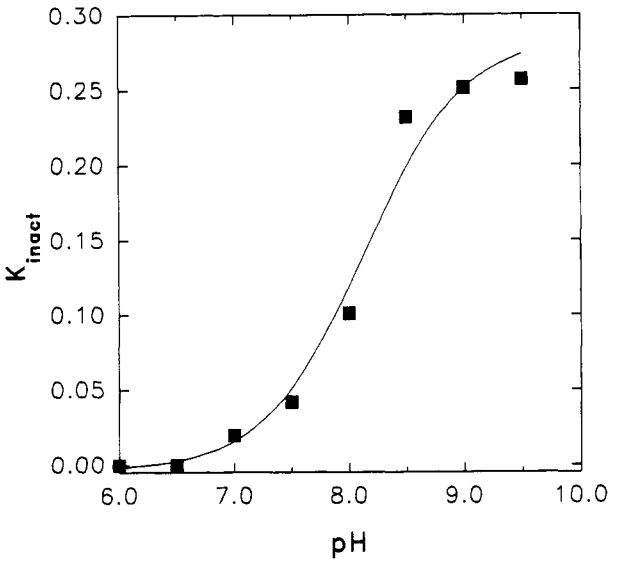

FIGURE 5: pH dependence of the rate of inactivation of L-aspartase by L-ASA. The line through the data is a fit to an equation for pH dependent activity changes (eq 4).

to yield a maximum rate of inactivation. Analysis of this pH-dependent inactivation by a fit to eq 4 yields a pK value of 8.1, and a maximum rate of inactivation with L-ASA of 0.29 min^{-1}. This pK value is consistent with the ionization of a cysteine as the enzyme group which is responsible for the nucleophilic attack on FAA.

DISCUSSION

Mode of Inactivator Binding. Both substrate and product analogues that contain an aldehydic group in place of the β-carboxyl group have been shown to bind to L-aspartase and to lead to irreversible inactivation. Protection studies were carried out against L-ASA and FAA inactivation in order to identify the nature of and the site of inactivation. Protection from L-ASA inactivation of L-aspartase showed that L-ASA is inactivating by binding at the active site of the enzyme. Complete protection requires the presence of both fumarate and Mg^{2+} (Table 1), supporting earlier studies which had shown that a divalent metal ion, and also an activator, are required for fumarate binding to occur at higher pH. Addition of AMA, a known activator of L-aspartase, does not enhance the protection that is provided by fumarate and Mg^{2+} alone, and activator and metal ion by themselves afford little protection against L-ASA inactivation. Thus, L-ASA must also be binding as an activator to the enzyme, analogous to the binding of L-aspartic acid.

In contrast to the nearly complete protection against L-ASA inactivation, only slight protection against inactivation by FAA is seen with fumarate and Mg^{2+}. This demonstrates the inability of this aldehydic product analogue to bind to the activator site. Addition of an activator, such as AMA or L-ASA, is required to see substantial protection. The addition of an activator and a divalent metal ion, in the absence of fumarate, results in an increase in the rate of inactivation by FAA. This shows that FAA is able to bind more tightly to the enzyme-activator–metal ion complex than to the free enzyme form. This is consistent with the cooperativity which has been observed in binding between the activator site and the active site of L-aspartase.

Mechanism-Based Inactivation. While several structural analogues have been identified that can bind to L-aspartase as competitive inhibitors, the enzyme has shown absolute substrate specificity for L-aspartic acid, and no alternative substrates have previously been identified (Falzone et al., 1988). The catalytic production of ammonium ion by L-aspartase in the presence of a aldehydic substrate analogue indicates that L-ASA must be acting as an alternative substrate to yield FAA and NH_4^+ as the products. In one catalytic event out of 30 this aldehydic product then reacts with a functional group at the active site to inactivate the enzyme.

The inactivation of L-aspartase proceeds at each concentration of L-ASA without an observable time lag, supporting the idea that FAA inactivates the enzyme prior to release. If a threshold concentration of free FAA was required to accumulate before subsequent binding would lead to inactivation, then a time lag would be observed before inactivation occurred. Attempts to trap any FAA that is being produced by enzymatic coupling with fumarase had no perceptible effect on the observed rate of inactivation.

Mechanism of Inactivation. The adduct that forms between L-ASA and L-aspartase has been proposed to arise *via* a Michael addition to carbon-2 of FAA, the deaminated product. This evidence is consistent with the electron-withdrawing effect of the carbonyl moiety of the aldehydic group, which would then make carbon-2 of FAA electron deficient and susceptible to nucleophilic addition. Preliminary studies have reported incorporation of label into the protein fraction of ASA-modified enzyme upon treatment with NaB^3H_4 (Yumoto et al., 1982). These results support the proposal that the aldehydic group has remained intact after covalent attachment to L-aspartase. Our proposed mechanism is further supported by the subsequent reaction of the free aldehyde of this enzyme adduct with 2,4-dinitrophenylhydrazine (DNPH) to yield a nondissociable hydrazone adduct (Figure 4). Analogous treatment of glucose 6-phosphate dehydrogenase by 4-hydroxy-2-nonenal, followed by derivatization with DNPH, led to the identification of an active site lysyl residue in this enzyme (Szweda et al., 1993).

The proposed mechanism of inactivation of L-aspartase by aldehydic product analogues predicts that the rate of enzyme inactivation would be enhanced by substituents that make carbon-2 more electron deficient. This prediction was tested by examining several fumarate analogues with different electron-withdrawing substituents (Table 2). The methyl ester of FAA leads to the inactivation of L-aspartase with a rate of inactivation which is nearly 5 orders of magnitude greater than that of FAA, indicating that as greater electron-withdrawing groups (with $COOCH_3 \gg COOH > CH_3$) are attached to these fumarate analogues at position R_2, the rate of inactivation is observed to increase (Baer & Urbas, 1970). For substituents that are located at the β-position to the proposed site of nucleophilic attack, the greater resonance stabilization provided by a nitro group, as compared to an

aldehyde, will also make this position more electron deficient. The methyl ester of β-nitroacrylate analogue shows the highest rate constant for inactivation, due to the highly electron-withdrawing ester and the additional stabilization of the Michael adduct by the β-nitro group.

Identification of an Enzyme Reactive Group. The p*K* value of 8.1 that has been observed for the inactivation of L-aspartase is consistent with that expected for a cysteine residue. In model studies, thiol groups were found to show a similar pH dependent reactivity toward α,β-unsaturated compounds and were also shown to be considerably more reactive than amino groups (Friedman et al., 1965). Chemical modification studies have previously suggested the importance of a cysteine for catalytic activity (Mizuta & Tokushige, 1975; Ida & Tokushige, 1985a). However, site-directed mutagenesis of a cysteine identified in this study (cysteine 430) did not confirm the essentiality of this functional group (Murase et al., 1991). Mutagenesis of a highly conserved cysteine, which is present throughout the fumarase–aspartase family of enzymes, also had no effect on the activity of L-aspartase (Saribas et al., 1994). However, tandem mass spectroscopy analysis of L-aspartase that has been inactivated with L-ASA has recently led to the identification of another cysteine residue that appears to be the enzyme nucleophile (F. Giorgianni and R. E. Viola, manuscript in preparation).

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