L-Aspartase from *Escherichia coli*: Substrate Specificity and Role of Divalent Metal Ions†

Christopher J. Falzone,‡ William E. Karsten, Judith D. Conley,§ and Ronald E. Viola*
Department of Chemistry, The University of Akron, Akron, Ohio 44325
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ABSTRACT: The enzyme L-aspartase from *Escherichia coli* has an absolute specificity for its amino acid substrate. An examination of a wide range of structural analogues of L-aspartic acid did not uncover any alternate substrates for this enzyme. A large number of competitive inhibitors of the enzyme have been characterized, with inhibition constants ranging over 2 orders of magnitude. A divalent metal ion is required for enzyme activity above pH 7, and this requirement is met by many transition and alkali earth metals. The binding stoichiometry has been established to be one metal ion bound per subunit. Paramagnetic relaxation studies have shown that the divalent metal ion binds at the recently discovered activator site on L-aspartase and not at the enzyme active site. Enzyme activators are bound within 5 Å of the enzyme-bound divalent metal ion. The activator site is remote from the active site of the enzyme, since the relaxation of inhibitors that bind at the active site is not affected by paramagnetic metal ions bound at the activator site.

L-Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible deamination of L-aspartic acid to form fumaric acid and ammonia. The enzyme from *Escherichia coli* is tetrameric, with a subunit molecular weight of 48 000 (Williams & Lartigue, 1967). L-Aspartase has been shown (Ida & Tokushige, 1985; Karsten et al., 1986) to possess an activator site that has the unusual specificity of binding the substrate L-aspartic acid. It has been observed that, at pH 8.0 and above, occupation of this site with aspartic acid or with certain structural analogues of the substrate eliminates the reaction time lags that are observed in the amination direction and the nonlinear kinetics seen in the deamination direction (Karsten et al., 1986).

L-Aspartase has been reported to be specific for its amino acid substrate L-aspartic acid, showing no activity with D-aspartic acid or crotonic acid (Virtanen & Ellfolk, 1955), glycine, alanine, glutamine, maleic acid, or glutaconic acid (Quastel & Woolf, 1926). In addition, L-cysteic acid, α,β-diaminosuccinic acid (Ellfolk, 1954), leucine, mesaconic acid, aconitic acid, sorbic acid or the diamide of fumarate (Virtanen & Tarnanen, 1932), phenylalanine, tyrosine, dioxyphenylalanine, histidine, or the mono- and diethyl esters of fumarate (Jacobsohn & Soares, 1936) have all failed to show turnover. Also, inhibition studies have reported that citrate, EDTA,[1] and pyrophosphate block the action of L-aspartase (Ellfolk, 1953), as well as 1-propanol (Erkama & Virtanen, 1951).

The enzyme was previously observed to have an absolute requirement for a divalent metal ion activator at higher pH (Rudolph & Fromm, 1971), with some indication that L-aspartase may possess activity in the absence of divalent metal ions at low pH (Suzuki et al., 1973). Alkali earth metals such as Mg^{2+} and transition metals such as Mn^{2+} have been shown to provide some degree of activation (Wilkerson & Williams, 1961). Other metals, such as Be^{2+} and Ba^{2+} do not activate L-aspartase. It has recently been observed (W. E. Karsten and R. E. Viola, unpublished results) that the enzyme exists in a pH-dependent equilibrium between two forms. The higher pH form of L-aspartase is activated by divalent metal ions and substrate analogues, while the low-pH enzyme form does not require any effectors for catalytic activity.

In this paper, we examine the substrate specificity and the divalent metal ion stoichiometry and specificity of L-aspartase. Paramagnetic relaxation studies have allowed a role to be proposed for divalent metal ions in the mechanism of L-aspartase.

Experimental Procedures

Materials. L-Aspartic acid and all substrate analogues used in these studies were purchased from Sigma Chemical Co., except for the following compounds: 2-amino-4-phosphonobutyric acid and 2-amino-3-phosphonopropionic acid were purchased from CalBiochem, fumaric acid was from Eastman, 2-bromosuccinic acid was from Alfa Products, and 3-nitropropionate was from Aldrich Chemical Co. Buffers and all other chemicals were of reagent grade. L-Aspartase was purified from *E. coli*, strain B, according to the dye–ligand chromatography method of Karsten et al. (1985).

Methods. Assays were performed at 30 °C in thermostated 1-cm path-length cells with a single-beam Perkin-Elmer Lambda-1 spectrophotometer. Inhibition constants were estimated according to the method of Waley (1982) or were determined by varying the inhibitor concentrations from $0.5K_m$ to $2–4K_m$ and fitting the data by using a BASIC adaptation of the computer programs of Cleland (1979).

Relaxation time measurements for ^{13}C and ^{31}P nuclei were obtained by either a 180°–τ–90° or a 90°–τ–90° pulse sequence. T_1 values were calculated by using nonlinear regression methods (Gerhards & Dietrich, 1976). All ^{13}C (100-MHz observation frequency) and ^{31}P (161-MHz) spectra were obtained on a Varian XL-400 at 7 ± 1 °C in 10 mM sample tubes with a total sample volume of about 1.5 mL. Samples were centered in the receiver coil of the probe and maintained in position by using a vortex suppression plug. Exponential line broadening of approximately 1 Hz was ap-

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‡ Present address: Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.
§ Present address: Department of Chemistry, University of Houston, Houston, TX 77004.

[1] Abbreviations: 2,3-DPG, 2,3-diphosphoglycerate; AA, atomic absorption; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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plied prior to Fourier transformation to enhance the signal to noise ratios. Digital resolution of at least 0.5 Hz and spectral windows of about 5000 Hz were utilized to minimize the effects of pulse imperfections. Enzyme solutions for each set of experiments were identical, except for the identity of the divalent metal ion, which was Mn^{2+} as the paramagnetic probe or Mg^{2+} as the diamagnetic control. Solutions generally contained 0.2–0.5 mM L-aspartase (subunit concentration), metal ions present at 90–95% of this concentration, and 100–200 mM substrate analogue or activator in Hepes buffer, pH 7–7.5.

Relaxation in the presence of paramagnetic probes has been adequately reviewed in the literature (Mildvan & Cohn, 1970; Dwek, 1973). In this study, paramagnetic divalent metal ion–substrate analogue nuclear distances were determined by measuring spin–lattice relaxation rates of the inhibitor nuclei in the presence of Mn^{2+}. In the fast-exchange limit, these relaxation times yield the paramagnetic contribution to the relaxation rate ($1/T_{1p}$) and may be used to calculate the distance r in the Solomon–Bloembergen equation (Solomon, 1955; Bloembergen, 1957) if the value of τ_c is determined.

Manganese binding studies were conducted on a Varian Model V4500 EPR spectrometer that has been extensively modified and updated. Solutions of L-aspartase (~1.5 mg/mL) in buffered solutions from pH 6 to pH 9 were titrated with Mn^{2+}. Measured EPR signal intensities were converted to free metal concentrations by comparison to standard curves, and the dissociation constants were calculated by Scatchard analysis (Scatchard, 1949).

Protein determinations were made by using the standard Biuret technique (Gornall et al., 1949). Formation of the exchange inert enzyme–metal ion complexes was accomplished by adding excess (1.5 mM) Co^{2+} to a solution of L-aspartase. After incubation, oxidation of Co^{2+} to Co^{3+} was accomplished with either hydrogen peroxide or *m*-chloroperoxybenzoate (Ryzewski & Takahashi, 1975). Subsequent dialysis against buffer containing EDTA was utilized to remove all unbound metal ions. Manganese binding and cobalt binding to L-aspartase were determined by atomic absorption spectroscopy on a Jarrell-Ash Model 810 spectrometer. Aliquots were removed from the enzyme solutions before and after dialysis and examined to determine total and bound metal, respectively.

RESULTS

Substrate Analogues. A survey was conducted to identify possible inhibitors or alternate substrates of the deamination reaction catalyzed by L-aspartase. An examination of potential inhibitors not only can provide important information about the structural requirements that L-aspartase places on the amino acid substrate but also can identify analogues that can be utilized for paramagnetic relaxation studies. The results of these studies are summarized in Tables I and II, where Table I lists the variety of compounds that were found to be competitive inhibitors vs L-aspartic acid, with K_i values ranging from 0.2 to 36 mM. Table II is a list of the compounds that showed no inhibition of the L-aspartase-catalyzed deamination reaction. No analogues were identified that were alternate substrates for L-aspartase.

Metal Ion Requirements and Stoichiometry. L-Aspartase has previously been reported to have an absolute requirement for divalent metal ions above neutral pH (Rudolph & Fromm, 1971). However, enzyme purified by dye–ligand chromatography as the last step (Karsten et al., 1985) retained over 50% of the original activity observed at pH 7 when dialyzed extensively in the presence of chelators. Atomic absorption spectroscopy of the dialyzed enzyme confirmed the absence of various divalent metal ions, ruling out the possibility that metal ions have not been completely removed under the dialysis conditions.[2]

We have surveyed numerous divalent metal ions to determine the binding specificity of the enzyme and the activation

Table I: Inhibitors of L-Aspartase[a]

analogue	structure[b] R_1	structure[b] R_2	K_i[c] (mM)
O-phospho-D-serine	NH_3^+	OPO_3^{2-}	0.20
D-malate	OH	COO^-	0.66
DL-2-amino-3-phosphono-propionate	NH_3^+	PO_3^{2-}	0.66
3-nitropropionate	H	NO_2	0.83
2,3-diphosphoglycerate	OPO_3^{2-}	OPO_3^{2-}	1.1
mercaptosuccinate[d]	SH	COO^-	1.6
L-2-chlorosuccinate	Cl	COO^-	1.7
DL-2-bromosuccinate	Br	COO^-	2.3
DL-2-amino-4-phosphono-butyrate	NH_3^+	$CH_2PO_3^{2-}$	2.4
D-2-methylmalate[e]	OH	COO^-	3.7
2-hydroxy-3-nitro-propionate	OH	NO_2	6.7
N-acetyl-L-aspartate	$NHC(O)CH_3$	COO^-	6.7
(1-aminopropyl)-phosphonate[f]	NH_3^+	CH_3	17
β-aspartylhydrazine	NH_3^+	$C(O)NHNH_2$	18
methylsuccinate	CH_3	COO^-	23
phosphoglycolate	H	OPO_3^{2-}	23
succinate	H	COO^-	24
L-malate	OH	COO^-	31
(aminomethyl)-phosphonate[g]	NH_3^+	*g*	33
(3-aminopropyl)-phosphonate[h]	NH_3^+	$CH_2PO_3^{2-}$	36

[a] Assay conditions: 100 mM Hepes, pH 7.0, 2 mM L-aspartic acid, and variable inhibitor concentration. [b] Parent structure: $R_2CH_2CH(R_1)COO^-$. [c] Average standard error on K_i values is ±10%. [d] Time-dependent inhibition was observed with this analogue. The K_i value measured under initial velocity conditions is reported. [e] α-Proton replaced by an α-methyl group. [f] α-Carbonyl group replaced by α-phosphonate. [g] $-CH_2COO^-$ side chain of aspartic acid replaced by $-PO_3^{2-}$. [h] α-Carboxyl replaced by a proton.

Table II: Noninhibitors of L-Aspartase[a]

β-alanine	D- or L-glutamate
D- and L-alanine	L-homoserine
(1-aminoethyl)phosphonate	DL-*threo*-β-hydroxyaspartate
L-2-amino-1-propanol	hydroxybutyrates or -propionates
D- or L-asparagine	itaconate
D-aspartate	α-ketoglutarate
L-aspartate esters	lactate
DL-γ-carboxyglutamate	α-methyl-DL-aspartate
chloro- or phosphonoacetate	D- or L-*N*-methylaspartate
citrate	L-2-methylmalate
L-cysteine or serine	oxalate
2,2-difluorosuccinate	L-serine *O*-sulfate

[a] These compounds showed less than 5% inhibition of L-aspartase when examined in 100 mM Hepes, pH 7.0, 2 mM L-aspartic acid, and analogue concentrations of at least 10 mM.

Table III: Activation of L-Aspartase by Divalent Metal Ions[a]

metal ion	K_a (μM)	metal ion	K_a (μM)
Zn	0.21 ± 0.04	Co	2.0 ± 0.3
Cd	0.7 ± 0.5	Mg	4.9 ± 2.4
Mn	0.8 ± 0.4	Ca	19 ± 4

[a] Assay conditions were 100 mM Hepes, pH 7.0, and 30 mM L-aspartic acid.

[2] Atomic absorption spectroscopy indicated that the dialyzed enzyme did not contain detectable levels of the following metal ions: Ca, Cd, Co, Cu, Fe, Mo, and Ni. The maximum levels detected for the following metal ions were Mg, 0.05 mol/mol of subunit; Mn, 0.009; and Zn, 0.04.

Table IV: Calculated Distances for the Mn^{2+}–Activator–L-Aspartase Complex[a]

activator	distance (Å)				
	C-1	C-2	C-3	C-4	P
O-phospho-D-serine	3.6	4.6	4.0		5.6
3-nitropropionate	3.7	5.1	5.1		
D-aspartate	3.3	3.5	3.8	3.2	

[a] Solutions contained 100–200 mM activator, 0.2–0.5 mM L-aspartase, and divalent metal ions at 90–95% of the enzyme concentration, in Hepes buffer, pH 7–7.5, in a total volume of 1.5 mL.

properties of various metal ions. A number of different metal ions are capable of activating L-aspartase (Table III) at neutral pH, with K_a values ranging from about 0.2 to 20 μM.

The divalent metal ion stoichiometry of L-aspartase was determined by AA spectroscopy of the exchange-inert Co^{3+}–enzyme complex. When the enzyme was incubated with excess Co^{2+} and then dialyzed after oxidation, 0.86 mol of metal ion remained bound per mole of enzyme subunits. When the binding stoichiometry was determined in competition with Mn^{2+}, with both metal ions initially present in excess at their K_a ratios, a reduction in the number of Co^{3+} ions bound per subunit to 0.52 was observed. The stoichiometry for Mn^{2+} was also measured directly by AA spectroscopy, and 0.80 metal ion is bound per subunit. Subsequent dialysis against buffer containing EDTA removed virtually all of the Mn^{2+} bound to the enzyme. The binding of Mn^{2+} to L-aspartase was also examined by EPR spectroscopic studies. K_d values were determined to range from about 200 μM at pH 6 to 6 μM at pH 9.

Magnetic Resonance Studies. Spin–lattice relaxation times for ^{13}C nuclei of substrate analogues interacting with L-aspartase were measured in solutions containing high concentrations (0.2–0.5 mM) of enzyme and either the bound paramagnetic manganous ion or the diamagnetic magnesium ion as a control. Table IV shows the distances calculated from the Solomon–Bloembergen equation for the Mn^{2+}–enzyme solutions of *O*-phospho-D-serine, 3-nitropropionate, and D-aspartate. The distances observed from the enzyme-bound metal ion to the carbons of D-aspartic acid are comparable to those of the other analogues that were examined. This was unexpected because D-aspartate is a good activator of the enzyme, but was shown not to bind at the active site. This raises the question of the location of the divalent metal ion binding site. Divalent metal ion binding may be taking place at both the active site and the activator site, or only at the activator site. To test this hypothesis, paramagnetic relaxation rates were measured in solutions that contained both an activator and fumarate, one of the substrates of the amination reaction. As can be seen from Table V, relaxation rates for the activators (2-amino-4-phosphonobutyrate or D-aspartate) are perturbed by the presence of paramagnetic metal ions relative to the diamagnetic control. However, for the fumarate present in the same samples, no apparent interaction with the enzyme-bound metal ion was detected.

To further investigate the location of the divalent metal ion, ^{31}P relaxation rates were measured for 2,3-DPG, a structural analogue of L-aspartic acid that does not bind at the activator site. The relaxation rates observed for both the diamagnetic and paramagnetic enzyme solutions (15 s^{-1}) were identical within experimental error.

Table V: Relaxation Rate Data for Enzyme Solutions Containing Fumarate, Inhibitor, and either Mn^{2+} or Mg^{2+}[a]

expt	inhibitor and substrate	M^{2+}	T_1 values (s)				
			C-1	C-2	C-3	C-4	P
1	D-aspartate	Mg	16.0	3.2	1.6	nd	
		Mn	1.6	2.2	1.2	nd	
	plus fumarate	Mg	nd	4.1[b]		nd	
		Mn	nd	5.5[b]		nd	
2	2-amino-4-phosphono-butyrate	Mg	9.0	nd	3.1	4.2	4.8
		Mn	7.8	nd	1.1	0.4	2.7
	plus fumarate	Mg	nd	3.3[b]		nd	
		Mn	nd	3.3[b]		nd	

[a] Solutions contained 125 mM activator, 90 mM fumarate, 0.2–0.5 mM L-aspartase, and divalent metal ions at 90–95% of the enzyme concentration, in Tris buffer, pH 7.3, in a total volume of 2 mL. [b] Average T_1 value for carbons 2 and 3 of fumaric acid.

DISCUSSION

Substrate Binding Specificity. Binding of substrate analogues at the active site of L-aspartase requires the presence of a carboxylate or the functional equivalent of a carboxylate group in the 1- and 4-positions. Substitution with a phospho, phosphono, or nitro group can fulfill this requirement at the 4-position. Analogues that lack this functionality, such as L-alanine or L-serine (missing β-carboxyl) or (1-aminoethyl)phosphonate (missing α-carboxyl), do not show inhibition (Table II).

The requirement for the amino group at the α position is not as stringent as the necessity for both of the carboxyl groups. Diminished affinity is observed when the amino group is absent, such as with succinate. However, even substitution by a phosphate group, as in 2,3-DPG, or a halogen (bromo- or chlorosuccinate) results in significant inhibition. Interestingly, for 3-nitropropionate, where the amino group is missing and a nitro group is substituted for one of the carboxyls, one of the best inhibitors is observed. Inhibition of L-aspartase by this compound has been proposed to result from the formation of a carbanion species at carbon-3 on ionization that is transition state like or a metastable intermediate (Porter & Bright, 1980).

All of the compounds discussed above have the L configuration if carbon-2 is chiral. However, some D isomers have shown significant inhibition of L-aspartase. While D-aspartate is not an inhibitor (Table II), D-malate and *O*-phospho-D-serine are excellent inhibitors of L-aspartase. This indicates that substituents at the α-position in the D configuration are responsible for some increased affinity for the enzyme. The binding orientation of these analogues at the active site has not been established; however, these compounds are competitive inhibitors of L-aspartase but are not substrates. No substrate activity has been observed for any of the structural analogues of L-aspartic acid that have been examined.

The inhibition of L-aspartase that had previously been observed with citrate, EDTA, and pyrophosphate (Ellfolk, 1953) has been found to be due to the ability of these compounds to chelate the divalent metal ion that is required for enzyme activity at higher pH. When sufficient divalent metal ion is added to account for metal ion binding to citrate, no inhibition of L-aspartase is observed by these compounds.

Metal Ion Specificity. There have been several reports on the divalent metal ion specificity and activation of L-aspartase (Wilkinson & Williams, 1961; Ichihara et al., 1955). In marked contrast to the absolute substrate specificity of the enzyme, the specificity of L-aspartase for metal ions is low, and many different divalent metal ions are capable of activation. Transition metals have been found to be better activators than the alkali earth metals, with K_a values in the range of 0.2–2 μM for transition metal ions as compared to >5 μM for the alkali earth metals (Table III). Competitive binding studies have indicated that the binding of Mn^{2+} and

Co^{2+}, and probably the binding of all the divalent metal ions examined, is occurring at a common divalent metal ion site on L-aspartase. Transition metals have also been found to inhibit the enzyme at concentrations above 100 μM, whereas no significant inhibition was observed with the alkali earth metals. The inhibition by transition metal ions is probably the result of weaker, nonspecific binding to L-aspartase.

EPR measurements have shown that tighter binding of Mn^{2+} is observed at higher pH, indicating that the low-pH form of L-aspartase has little affinity for the divalent metal ion. These observations are consistent with our recent studies on the pH-dependent activity changes of L-aspartase (W. E. Karsten and R. E. Viola, unpublished results) that have shown that the absolute requirement for divalent metal ions only occurs at higher pH (>7). Significant activity is observed at lower pH in the absence of metal ions. Previous kinetic studies have been carried out at pH 8.5–9.0 where the enzyme activity is extremely low in the absence of divalent metal ions. Under these conditions, the requirement for a divalent metal ion would appear to be absolute.

The nonspecific nature of the metal ion activation of L-aspartase implies a binding role for the metal ion rather than involvement as an acid–base catalyst. The role of the divalent metal ion was further explored by an examination of the location of the metal ion binding site by magnetic resonance studies.

Magnetic Resonance Studies. The value of τ_c was estimated to be about 1×10^{-8} s in order to yield relative ^{13}C metal ion distances of the substrate analogues. This τ_c value is based on τ_s, which is calculated from the Bloembergen–Morgan equation (Bloembergen & Morgan, 1961) with a value for τ_v of 5×10^{-13} (Reuben & Cohn, 1970), and on τ_r values, which were estimated by comparison to comparable molecular weight complexes. In the case of *O*-phospho-D-serine and 3-nitropropionate, the analogues appear to be interacting with the metal ion through the C-1 carboxyl end with no other group interacting directly. The monodentate nature of these analogues is in contrast with the bidentate binding of *O*-phosphoserine and L-aspartate to Mn^{2+} observed in the absence of enzyme (Khazaeli & Viola, 1984) In fact, in these enzyme-free studies, the phosphate–metal distances were observed to be the shortest. D-Aspartate seems to have a bidentate interaction with Mn^{2+} through both carboxyl groups. Paramagnetically induced relaxation of this noninhibitory activator indicates that the metal ion cannot be binding exclusively at the active site. These relaxation studies were repeated in the presence of a substrate analogue that binds at the activator site (either 2-amino-3-phosphonopropionate or D-aspartate) and a substrate that binds exclusively at the active site (fumarate). Paramagnetically induced relaxation was observed for the carbons and phosphorus of the analogues, but no enhanced relaxation was observed for the carbon atoms of fumarate. These results indicate that divalent metal ions are bound at a site remote from the fumarate binding site, and therefore remote from the active site.

The substrate analogue 2,3-DPG is an excellent inhibitor, but it is one of the few competitive inhibitors tested not to show activating capabilities. Therefore, the readily observed ^{31}P nuclei should not have their relaxation rates enhanced by manganous ion if the divalent metal ion is binding exclusively at the activator site. The ^{31}P relaxation rates for 2,3-DPG interacting with L-aspartase were identical with either Mn^{2+} or Mg^{2+} bound to the enzyme. This evidence, along with the relaxation data for the activators and fumarate, indicates that the divalent metal ion binds with the activator in the higher pH realm where both are required for activity. This site must be well separated from the active site that is present on the same enzyme subunit, as well as from the active site on an adjacent subunit. Distances of less than 10–12 Å between these sites would result in an observable paramagnetic enhancement of the relaxation rates of substrates or analogues bound at the active site.

The presence of metal ions is known to produce changes in binding specificity. For example, the binding of ADP to the active site of adenylate kinase is directed to one of two nucleotide sites by the presence of a divalent metal ion (Rhoads & Lowenstein, 1968; Nageswara Rao et al., 1978). However, as far as we are aware, this is the first case reported where a metal ion has directed the partitioning of binding of a compound between two spatially and functionally distinct sites on an enzyme.

Registry No. Zn, 7440-66-6; Cd, 7440-43-9; Mn, 7439-96-5; Co, 7440-48-4; Mg, 7439-95-4; Ca, 7440-70-2; L-aspartase, 9027-30-9; *O*-phospho-D-serine, 73913-63-0; D-malate, 636-61-3; DL-2-amino-3-phosphonopropionate, 20263-06-3; 3-nitropropionate, 504-88-1; 2,3-diphosphoglycerate, 138-81-8; mercaptosuccinate, 70-49-5; L-2-chlorosuccinate, 4198-33-8; DL-2-bromosuccinate, 584-98-5; DL-2-amino-4-phosphonobutyrate, 20263-07-4; D-2-methylmalate, 6236-10-8; 2-hydroxy-3-nitropropionate, 36629-54-6; *N*-acetyl-L-aspartate, 997-55-7; (1-aminopropyl)phosphonate, 14047-23-5; β-aspartylhydrazine, 13010-39-4; methylsuccinate, 498-21-5; phosphoglycolate, 13147-57-4; succinate, 110-15-6; L-malate, 97-67-6; (aminomethyl)phosphonate, 1066-51-9; (3-aminopropyl)phosphonate, 13138-33-5; β-alanine, 107-95-9; D-alanine, 338-69-2; L-alanine, 56-41-7; (1-aminoethyl)phosphonate, 6323-97-3; L-2-amino-1-propanol, 2749-11-3; D-asparagine, 2058-58-4; L-asparagine, 70-47-3; D-aspartate, 1783-96-6; DL-γ-carboxyglutamate, 56271-99-9; chloroacetate, 79-11-8; phosphonoacetate, 4408-78-0; citrate, 77-92-9; L-cysteine, 52-90-4; L-serine, 56-45-1; 2,2-difluorosuccinate, 665-31-6; D-glutamate, 6893-26-1; L-glutamate, 56-86-0; L-homoserine, 672-15-1; DL-*threo*-β-hydroxyaspartate, 4294-45-5; itaconate, 97-65-4; α-ketoglutarate, 328-50-7; lactate, 50-21-5; α-methyl-DL-aspartate, 2792-66-7; D-*N*-methylaspartate, 6384-92-5; L-*N*-methylaspartate, 4226-18-0; L-2-methylmalate, 6236-09-5; oxalate, 144-62-7; L-serine *O*-sulfate, 626-69-7.

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Inactivation of Dopamine β-Hydroxylase by *p*-Cresol: Isolation and Characterization of Covalently Modified Active Site Peptides

Walter E. DeWolf, Jr.,*,‡ Steven A. Carr,‡ Angela Varrichio,‡ Paula J. Goodhart,‡ Mary A. Mentzer,‡ Gerald D. Roberts,‡ Christopher Southan,§ Roland E. Dolle,§ and Lawrence I. Kruse§

Departments of Medicinal Chemistry, Physical and Structural Chemistry, and Molecular Pharmacology, Smith Kline & French Laboratories, Research and Development Division, King of Prussia, Pennsylvania 19406, and Department of Medicinal Chemistry, Smith Kline & French Research Ltd., The Frythe, Welwyn, Herts, U.K. AL6 9AR

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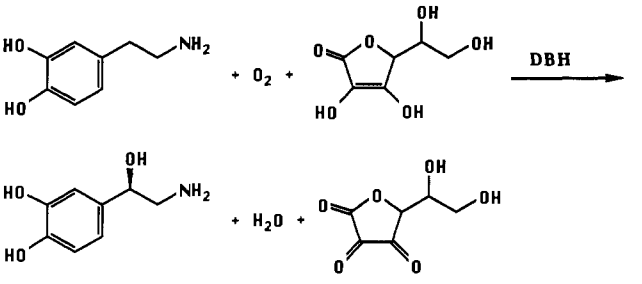
ABSTRACT: Recently, *p*-cresol has been shown to be a mechanism-based inhibitor of dopamine β-hydroxylase (DBH; EC 1.14.17.1) [Goodhart, P. J., DeWolf, W. E., Jr., & Kruse, L. I. (1987) *Biochemistry 26*, 2576–2583]. This inactivation was suggested to result from alkylation of an active site residue by an aberrant 4-hydroxybenzyl radical intermediate. In support of this hypothesis, we report here the isolation and characterization of two modified tryptic peptides from DBH inactivated by *p*-cresol. Using a combination of automated Edman sequencing, mass spectroscopy (MS), and tandem MS, we have determined the sequence of the putative active site peptides, identified the site of attachment of *p*-cresol, and defined the chemical nature of the adduct formed. Both modified peptides are the same primary sequence: Ala-Pro-Asp-Val-Leu-Ile-Pro-Gly-Gln-Gln-Thr-Thr-Tyc-Trp-Cys-Tyr-Val-Thr-Glu-Leu-Pro-Asp-Gly-Phe-Pro-Arg, where Tyc is an amino acid residue with the in-chain mass of a cresol–Tyr adduct (106 + 163 Da). Gas-phase deuterium exchange studies (employing N^2H_3-DCI MS) of the isolated phenylthiohydantoin (Pth) derivatives of modified residue 13 demonstrate that *p*-cresol forms two chemically distinct covalent adducts and support the hypothesis that a (4-hydroxyphenyl)methyl radical is generated during catalysis. Rearrangement to a (4-methylphenyl)oxy radical may also occur prior to inactivation.

Dopamine β-hydroxylase (DBH;[1] EC 1.14.17.1) is a copper-containing mixed-function oxidase that catalyzes the hydroxylation of dopamine to norepinephrine (Scheme I; Levin et al., 1960; Skotland & Ljones, 1979; Rosenberg & Lovenberg, 1980; Villafranca, 1981; Ljones & Skotland, 1984). Despite its key physiological role in the biosynthesis of neurotransmitters and its absolute stereochemical specificity for the *pro-R* hydrogen (Battersby et al., 1976), DBH displays a remarkable lack of specificity toward organic substrates. In addition to hydroxylating a number of substituted phenethylamines and phenylpropylamines (Creveling et al., 1962), DBH has been shown to catalyze sulfoxidation of phenyl thioethers (May & Phillips, 1980; May et al., 1981), epoxidation of styrenes, and N-dealkylation of methylamines (Padgette et al., 1985). In recent years, the indiscriminate nature of DBH has led to numerous reports of mechanism-based inhibitors; for an extensive review, see Fitzpatrick and Villafranca (1987). Various speculative chemical mechanisms have been advanced to account for the observed inactivation. These implicitly assume the involvement of the latent elec-

Scheme I



* Address correspondence to this author at L-421, Smith Kline & French Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939.
‡ Smith Kline & French Laboratories.
§ Smith Kline & French Research, Ltd.

[1] Abbreviations: Da, dalton(s); DBH, dopamine β-hydroxylase; DCI, desorptive chemical ionization; DPM_T, total disintegrations per minute; FAB, fast atom bombardment; FABMS, fast atom bombardment mass spectroscopy; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; NEMAc, *N*-ethylmorpholineacetate; Pth, phenylthiohydantoin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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