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β-CYANOALANINE AS A SUBSTRATE FOR ASPARAGINASE. STOICHIOMETRY, KINETICS, AND INHIBITION

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SUMMARY

L-Asparaginases from guinea pig serum and *Escherichia coli* catalyze the hydrolysis of L-β-cyanoalanine in aqueous solution. The disappearance of β-cyanoalanine is paralleled by the formation of aspartic acid and NH_3 in the expected stoichiometric amounts. The *Escherichia coli* enzyme has a K_m of 12 mM for L-β-cyanoalanine and 0.011 mM for L-asparagine. The apparent V for β-cyanoalanine is about three-tenths that for asparagine. L-Aspartic acid and D-asparagine strongly inhibit the hydrolysis of β-cyanoalanine. L-Asparagine inhibits in a reversible, competitive manner and in the process is converted into aspartic acid (K_i = 1.5 mM). D-Aspartic acid, D-β-cyanoalanine, and carbobenzoxy-L-asparagine inhibit less effectively.

INTRODUCTION

As part of an exploratory study on the biosynthesis of asparagine from β-cyanoalanine, we reported earlier that guinea pig serum asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) catalyzes a slow but complete hydrolysis of L-β-cyanoalanine to aspartic acid and NH_3. When we attempted in an inverse isotope dilution experiment to determine whether or not this reaction proceeded through asparagine as an intermediate, we found that addition of asparagine inhibited the enzymatic hydrolysis of β-cyanoalanine to aspartic acid. A preliminary account of this work has appeared[1]. Recent application of *Escherichia coli* asparaginase as a chemotherapeutic agent has led us now to examine the relation of β-cyanoalanine to asparaginase from this source.

The bacterial enzyme likewise uses L-β-cyanoalanine as a substrate thus catalyzing Reaction 1.

$$CN\text{-}CH_2\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{-}COOH \longrightarrow COOH\text{-}CH_2\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{-}COOH + NH_3 \qquad (1)$$

Kinetic constants and the pH–activity curve for Reaction 1 and for the deamidation

of asparagine have been determined to compare the affinities of the enzyme for the two substrates and their rates of hydrolysis. A number of related substances have been tested as inhibitors of the nitrilase activity. Most effective of these are L-aspartic acid, the product, and D-asparagine, a substrate under other conditions. L-Asparagine (L-aspartic acid) inhibits competitively. The effects of L-β-cyanoalanine and L-aspartic acid on the hydrolysis of asparagine also have been tested. Since both substrates give the same products, microradioassays were used with L-β-cyano[4-^{14}C]alanine in the presence and absence of unlabeled asparagine or other additives, and with L-[^{14}C]-asparagine in the presence and absence of unlabeled β-cyanoalanine or L-aspartic acid. Radioactivity in aspartic acid was determined in each case.

MATERIALS AND METHODS

L-β-Cyano[4-^{14}C]alanine and nonisotopic L- and D-β-cyanoalanine had been prepared as described[2,3]. L-[^{14}C]Asparagine (uniformly labeled) (40.2 mC/mmole) was purchased from Nuclear-Chicago, Des Plaines, Ill.; L-asparagine·H_2O, from Mann Research Laboratories, New York, N.Y.; D-asparagine·H_2O, from Nutritional Biochemical Corp., Cleveland, Ohio. *E. coli* asparaginase (EC 3.5.1.1, L-asparagine amidohydrolase, "L-asparaginase EC-2", 22 I.U./mg), Preparation A, was purchased from Worthington Biochemical Corporation, Freehold, N.J. and used in most experiments. For determination of stoichiometry crystalline *E. coli* B asparaginase also was used. This was obtained from Dr. R. G. Denkewalter of Merck and Co., Inc., Rahway, N.J. It had an initial activity of 266 units/mg and contained only traces of metal, Preparation B. Guinea pig serum asparaginase was partially purified as described[4] and used after the treatment with calcium phosphate gel; it contained 1.53 mg of nitrogen per ml.

Stoichiometry was determined on the Beckman–Spinco automatic amino acid analyzer, model 120 (ref. 5). Systems for β-cyanoalanine, asparagine, aspartic acid, and NH_3 are given elsewhere[6].

Enzyme assays

E. coli asparaginase was diluted to 1 mg/ml with 0.85% NaCl and then with 0.1 M Tris–HCl buffer, pH 8.5. All other solutions were in buffer. Potency was determined with 0.1 mM L-asparagine; other concentrations of substrates are given as they were used. Incubation mixtures (150 μl) consisted of substrate (25 μl), enzyme (25 μl), and buffer (100 μl). The substrate and enzyme were preincubated separately for 5 min at 38° before mixing. In competition experiments, inhibitor and substrate were preincubated together for 5 min before addition of enzyme. Reaction mixtures were incubated for 2 min at 38° with 0.015 μg of Enzyme A for L-asparagine substrate and for 15 min with 4.2 μg of Enzyme A for β-cyanoalanine substrate followed by 4 min in a boiling water bath. Samples of 20 μl were electrophoresed on Whatman No. 1 paper for 4 h in pyridinium acetate buffer (pH 5.7) at 9 V/cm. Regions containing aspartic acid and neutral material were cut as strips (2 inch × 1 inch), which were counted as cylinders in 15 ml of "Liquifluor" (New England Nuclear Corp., Boston, Mass.) in a Nuclear–Chicago 722 liquid scintillation system. Correction was made for spontaneous hydrolysis to aspartic acid. The fraction found in aspartic acid of the counts/min electrophoresed times the total μmoles of substrate incubated and divided

by the reaction time gave the reaction velocity. Approx. 15% hydrolysis of both substrates took place under the conditions given. At a concentration of 14 mM β-cyanoalanine, and at 13.4 and 26.8 μM L-asparagine, rates of hydrolysis were linear within the 15- and 2-min-assay periods, respectively. Counts/min in aspartic acid of the uninhibited reaction *minus* that of the inhibited reaction divided by that of the uninhibited reaction and multiplied by 100 gave the percentage inhibition.

TABLE I

STOICHIOMETRY OF β-CYANOALANINE HYDROLYSIS AND ASPARTIC ACID AND NH_3 FORMATION CATALYZED BY ASPARAGINASE

For Reaction 1, 100 μl of enzyme preparation, 0.4 unit, was used in 400 μl of 0.03 M sodium borate buffer; for Reaction 2, 125 μg of enzyme Preparation A, 2.8 units, in 450 μl of 0.083 M Tris–HCl; for Reactions 3 and 4, 10.5 and 36.7 μg of crystalline enzyme Preparation B, 1.6 and 5.5 units, incubated as in Reaction 2, all at pH 8.5. Reactions 1 and 2 were stopped by placing them in a 100° bath for 4 min; Reactions 3 and 4, by adding an equal volume of 10% trichloroacetic acid.

*Experiment**	*Concentration change (mM) during incubation (h)*							*Stoichiometry (μmoles formed per μmole of β-cyanoalanine degraded)*
	0	*1*	*6*	*7*	*7.5*	*10*	*24*	
Guinea pig serum asparaginase, 0.4 unit/mg								
1. β-Cyanoalanine present	10.0	7.00			2.90		0.60	
β-Cyanoalanine degraded		3.00			7.10		9.40	
Aspartic acid formed		3.00			7.10		9.10	1
E. coli asparaginase, 22 units/mg								
2. β-Cyanoalanine present	26.6	16.5	2.60					
β-Cyanoalanine degraded		10.2	24.0					
Aspartic acid formed		9.9	23.6					1
E. coli asparaginase, crystalline, 149 units/mg								
3. β-Cyanoalanine present	27.2			6.64				
β-Cyanoalanine degraded				20.6				
Aspartic acid formed				20.5				1
Ammonia formed				19.4				1 (0.9)
4. β-Cyanoalanine present	28.6							
β-Cyanoalanine degraded						28.6		
Aspartic acid formed						28.6		1
Ammonia formed						24.4		1 (0.9)

* Under conditions of Reaction 2, D-asparagine was 75 and 100% deamidated in 1 and 6 h and D-β-cyanoalanine was qualitatively unchanged. Under conditions of Reaction 3, with 1.5 units, D-asparagine was 31%, and L-β-cyanoalanine was 17% hydrolyzed in 30 min.

RESULTS

Stoichiometry

Table I gives rates of hydrolysis of β-cyanoalanine under various conditions with partially purified preparations of asparaginase from guinea pig serum and *E. coli* and with a highly purified crystalline *E. coli* asparaginase. Disappearance of β-cyanoalanine corresponded closely to the appearance of aspartic acid. With the crystalline asparaginase 1 mole of aspartic acid and NH_3 were formed per mole of β-cyanoalanine degraded. No significant amount of asparagine was detected as a product of the

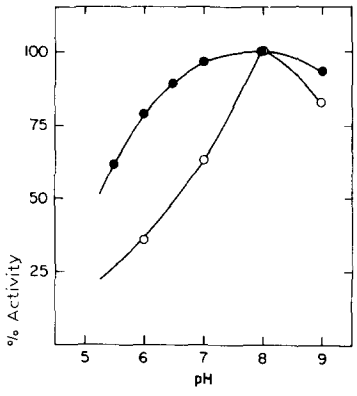


Fig. 1. The pH–activity curves for asparaginase hydrolysis of two substrates in 0.1 M Tris–maleate buffers. *E. coli* B asparaginase, 22 units/mg, was used. Enzyme and substrate concentrations were: 28 μg/ml asparaginase and 14 mM L-β-cyanoalanine (○—○); 0.1 μg/ml asparaginase and 11 μM L-asparagine (●—●).

nitrilase activity, even with smaller amounts of enzyme. Complete hydrolysis of β-cyanoalanine could be achieved, although the rate of hydrolysis declined markedly with time. Since the reaction is strongly inhibited by aspartic acid (*vide infra*), formation of this product probably contributes to the fall in rate.

L-Asparaginase and nitrilase activities as a function of pH

The pH optimum with both substrates was near 8.0. As observed previously in other buffers, L-asparagine deamidation had a broad pH range of activity with only a slight change in activity from pH 6 to 8.4 (ref. 7). L-β-Cyanoalanine hydrolysis had a narrower pH range; at pH 6 activity was 36% that at pH 8 (Fig. 1). Apparent ionization constants for L-β-cyanoalanine are pK_{a_1} 1.7 and pK_{a_2} 7.4; for L-asparagine, pK_{a_1} 2.1 and pK_{a_2} 9.0 (ref. 8). The high pH optimum suggests that the difference in the pH–activity curves on the acid side of neutrality is probably due less to the degree of ionization of the carboxyl group than of the protonated amino group.

Kinetic constants for L-asparagine and L-β-cyanoalanine as substrates

A double reciprocal plot of velocity and asparagine concentration gave a straight line. Calculated from the intercepts, K_m = 11 μM and V = 19.4 μmoles/min per mg of enzyme Preparation A (Table II). SCHWARTZ *et al.*[9] noted that the enzyme was unsaturated at 50 μM and saturated at 100 μM. After completion of this work, HO AND MILIKIN[10] reported that the K_m = 11.2 μM for crystalline *E. coli* B asparaginase containing Mg^{2+}. The derived K_m is considerably lower than that of guinea pig serum asparaginase: K_m = 2.2 mM at pH 9.6 in 0.05 M borate buffer[11]. However, it is well known that various asparaginases may differ in properties such as molecular weight, pH–activity range, and antitumor activity[7,9,12].

When β-cyanoalanine concentration was varied over a 37-fold range, a double reciprocal plot gave a straight line with K_m = 12 mM and V = 5.5 μmoles/min per mg (Table II). In the competition experiment with asparagine in which β-cyanoalanine concentration was varied over a 9-fold range, a double reciprocal plot for the uninhi-

TABLE II

COMPARATIVE V AND K_m FOR β-CYANOALANINE AND ASPARAGINE AS SUBSTRATES FOR *E. coli* L-ASPARAGINASE IN 0.1 M TRIS HCl BUFFER, pH 8.5

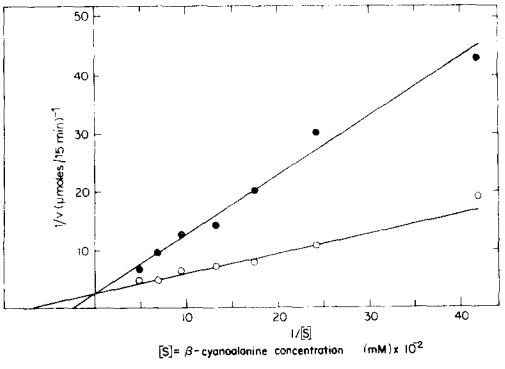
Enzyme, 22 units/mg, was 280 times more dilute for hydrolysis of asparagine than for that of β-cyanoalanine, and any inactivation during assay may not have been comparable for the two substrates. Concentration of β-cyanoalanine was varied by dilution of a constant amount of β-cyano [^{14}C]alanine with increasing amounts of carrier. Concentration of asparagine was varied largely by dilution of [^{14}C]asparagine with buffer. The specific activity of the available commercial asparagine and the low-saturating concentration of the enzyme did not for the most part permit addition of carrier. Kinetic constants therefore are based on the supplier's specific activity and their accuracy thus depends on this factor.

Substrate	V (*μmoles/min per 10 units* L-*asparaginase*)	*Apparent relative* V	K_m (*mM*)
L-β-Cyanoalanine			
(0.351–13.2 mM)	2.5	28	12
(2.41–21.1 mM)	2.6	30	11.5
L-Asparagine			
(1.27–29.3 μM)	8.8	100	0.011

bited reaction (Table II and lower curve in Fig. 2) gave K_m = 11.5 mM and V = 5.7 μmoles/min per mg.

Inhibition of β-cyanoalanine hydrolysis to aspartic acid

With β-cyanoalanine near its K_m concentration, L- and D-asparagine, L- and D-aspartic acid, D-β-cyanoalanine, and carbobenzoxy-L-asparagine were tested as inhibitors of nitrilase activity, some at over a 100-fold concentration range; Table III summarizes the results. Fig. 3 shows the increasing inhibition effected by increasing amounts of L-aspartic acid. Hydrolysis was inhibited 50% by 2.88 mM L-aspartic acid, or at the molar ratio of L-aspartic acid to β-cyanoalanine of 1:5.



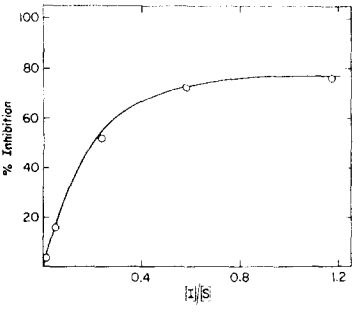


Fig. 2. Lineweaver–Burk diagram of the velocity of asparaginase-catalyzed hydrolysis of L-β-cyanoalanine to aspartic acid (○ - ○). The upper curve (●—●) shows the velocity of the reaction inhibited by 3.34 mM L-asparagine. *E. coli* B asparaginase, 22 units/mg, was used (see *Kinetic constants for* L-*asparagine and* L-β-*cyanoalanine as substrates*).

Fig. 3. Inhibition of asparaginase-catalyzed hydrolysis of L-β-cyano[4-^{14}C]alanine, 14.4 mM, with increasing amounts of L-aspartic acid, 0.133–8.33 mM (see Table III).

TABLE III

INHIBITION BY RELATED COMPOUNDS OF L-β-CYANOALANINE NITRILASE ACTIVITY OF *E. coli* ASPARAGINASE

L-β-Cyano[4-^{14}C]alanine (*S*), 2.1–2.2 μmoles, incubated with inhibitor (*I*) and 4.2 μg of asparaginase, 22 units/mg, in 150 μl for 15 min. Other conditions given under *Enzyme assays*. $[I_{50}]/[S]$ represents the ratio of the concentration of inhibitor to substrate at which enzyme activity was 50% inhibited; $[I]/[S]$ that ratio, at the percentage inhibition indicated.

Inhibitor (μmoles)	$[I_{50}]/[S]$	*Other inhibition studies*	
		$[I]/[S]$	*Inhibition (%)*
L-Asparagine (3.0–0.25)	0.26*	0.5	68
L-Aspartic acid (1.25–0.02)	0.20	0.5	71
D-Asparagine (4.5–0.04)	0.10	0.26**	73
		0.5	78
D-Aspartic acid (2.23–0.02)		0.1	9
		0.5	33
		1.06	47
D-β-Cyanoalanine		7.4	69
Carbobenzoxy-L-asparagine		2.1	30

* L-Asparagine underwent complete deamidation within first 2 min of incubation.
** D-Asparagine underwent 19% deamidation.

Inhibition was studied further at β-cyano[^{14}C]alanine concentrations which varied between 2.4 and 21 mM, with 3.34 mM L-asparagine, *i.e.* at a range of 35–85% inhibition. The Lineweaver–Burk plot (Fig. 2) shows competitive inhibition. Calculated from the intercept, $K_i = 1.54$ mM. L-Aspartic acid probably is the inhibiting species since, under conditions of 50% inhibition, L-asparagine is deamidated completely within the first 2 min of the 15-min reaction (Table III).

50% inhibition resulted at the molar ratio of D-asparagine to β-cyanoalanine of 1:10. The effect of D-asparagine could not be attributed to deamidation. D-Aspartic acid was a weaker inhibitor whose effect plateaued below 50% inhibition. When β-cyanoalanine hydrolysis was 73% inhibited by 3.46 mM D-asparagine, 19% hydrolysis of the latter resulted, which furnished an amount of D-aspartic acid that should account for only 4% inhibition (Table III).

Inhibition of asparagine hydrolysis to aspartic acid

With L-asparagine at its K_m concentration, 11 μM, under conditions described under *Enzyme assays*, L-β-cyanoalanine was tested as an inhibitor of asparaginase activity at molar ratios to asparagine from 5:1 to 2000:1. Asparaginase was inhibited by 37 and 20% with 0.01 and 0.02 M β-cyanoalanine. Below these concentrations, inhibition was not significant, and occasionally activation resulted that was variable and was not further pursued. L-Aspartic acid inhibited asparaginase by 64 and 93% at 0.01 and 0.1 M, but not at 1 mM.

DISCUSSION

The finding that partially purified preparations of asparaginase of different sources, mammalian and bacterial, catalyze the hydrolysis of β-cyanoalanine to

aspartic acid strongly suggests that this reaction is a property of asparaginase. The recent availability of *E. coli* B asparaginase in a highly purified crystalline state now allows β-cyanoalanine to be established as a true substrate of the enzyme. If the hydrolyses of asparagine and β-cyanoalanine are catalyzed at the same site, β-cyanoalanine presumably has a much lower affinity for it as judged by its much higher K_m value. Despite the 1000-fold difference in K_m, the rate of hydrolysis of β-cyanoalanine at V is only three-tenths that of L-asparagine. As indicated by both rates and K_m, asparagine clearly is the favored substrate. The β-carboxamide group of asparagine thus may be an important factor in the binding of this substrate to the protein.

Asparagine was not detected in any of the enzymatic hydrolyzates of β-cyanoalanine that were interrupted before completion, as in the kinetic runs summarized in Table I. However, its possible role as an intermediate in the conversion of β-cyanoalanine into aspartic acid is not ruled out by these observations since asparagine is hydrolyzed more rapidly than β-cyanoalanine.

D-Asparagine is a competitive inhibitor of L-asparaginase of crude extracts of various mycobacteria[13,14] and a substrate for the stereospecific D-asparaginase of a strain of *Brucella abortus*[15]. It is also a substrate for the partially purified[16] or crystalline *E. coli* L-asparaginase and is deamidated by the latter in 30 min at 27 mM at about 1.8 times the rate of β-cyanoalanine (Table I). D-Asparagine effectively inhibits the β-cyanoalanine nitrilase activity of *E. coli* L-asparaginase (50% at 1.4 mM, $[I]/[S] = 0.1$). L-Aspartic acid at concentrations near the K_m likewise inhibits both the nitrilase and L-asparaginase activities of this enzyme (0.01 M inhibits by 75 and 64%, respectively). L-β-Cyanoalanine at this concentration inhibits L-asparaginase activity by 37% in addition to acting as a substrate. Carbobenzoxy-L-asparagine, another known inhibitor of asparaginase[17], similarly inhibits nitrilase activity (30% at 29 mM).

These findings are all consistent with the possibility that the asparaginase and β-cyanoalanine nitrilase activities are catalyzed at the same site. The high $[I]/[S]$ ratios (1000) of L-aspartic acid and L-β-cyanoalanine required for significant inhibition of L-asparaginase probably reflect the greater affinity of the enzyme for asparagine than for these two other amino acids. JACKSON *et al.*[18] recently confirmed β-cyanoalanine as a substrate of asparaginase, and HANDSCHUMACHER *et al.*[16] established an additional substrate of *E. coli* asparaginase, diazo-4-oxo-L-norvaline, from which the enzyme catalyzes liberation of nitrogen. Since β-cyanoalanine inhibits the decomposition of diazo-4-oxo-L-norvaline[18], it seems likely that asparaginase, nitrilase, and diazo-4-oxo-L-norvaline decomposition are all catalyzed at the same site.

In the young leguminous plant *Lathyrus sylvestris* W., asparagine synthesis from β-cyanoalanine was recently found to take place without loss of cyano nitrogen[19], thus making unnecessary the concept of a direct role for asparaginase in the biosynthesis of asparagine from β-cyanoalanine in plants of this type. The strong inhibitory effect of aspartic acid on the asparaginase-catalyzed hydrolysis of β-cyanoalanine, however, could suggest a means of regulating the synthesis of asparagine. Thus, aspartic acid formed from asparagine by the action of L-asparaginase or by the metabolism of other substances might inhibit its own formation from β-cyanoalanine and thereby permit conversion of the latter into asparagine with preservation of cyano nitrogen. Probably asparagine itself, present in extremely high concentration (10%) in many young seedlings, also is serving this function of control, although no firm kinetic evidence

was obtained for asparagine as an inhibitor of nitrilase, since it underwent rapid deamidation at the concentration observed.

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