L-Asparaginase II of Escherichia coli

STUDIES ON THE ENZYMATIC MECHANISM OF ACTION*

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SUMMARY

The enzymatic mechanism of asparaginase action has been explored using the acyl acceptor, hydroxylamine. Asparaginase catalyzed the synthesis of the hydroxamate from asparagine and more slowly from aspartic acid. β-Aspartohydroxamate was also a substrate for asparaginase. These reactions have rates which are linearly dependent on the concentration of the enzyme and can therefore be used as convenient colorimetric assays. The activity which catalyzed these reactions migrated precisely with asparaginase during isoelectric focusing with an isoelectric point of 4.9. The reactions with hydroxylamine suggest a mechanism which involves a β-aspartyl enzyme intermediate. Further support for this mechanism has been obtained from exchange studies which showed that asparaginase catalyzed the incorporation of oxygen from 18O-labeled water into aspartic acid. Useful kinetic analysis of the hydrolysis of asparagine cannot be carried out because the concentration of water cannot be varied as a kinetic parameter. The hydroxylaminolysis reactions, however, allowed demonstration of a ping-pong mechanism consistent with a reaction pathway involving an acyl enzyme intermediate.

Since Lipmann and Tuttle (1, 2) first made use of hydroxylamine to trap activated carboxyl intermediates, the reagent has been used in the study of enzyme mechanisms. Meister et al. (3) showed that asparaginase partially purified from guinea pig serum catalyzed the formation of β-aspartohydroxamic acid at rates that were low in comparison to those of hydrolysis. By contrast, we have found that highly purified asparaginase II of Escherichia coli catalyzed this reaction relatively rapidly. Although asparaginase has been used for clinical and physiological studies, little is known about the mechanism of the reaction.

We thought it likely that the asparaginase reaction might involve covalent catalysis and thus be similar in mechanism to the proteases which form acyl enzyme intermediates. Jackson Cooney, and Handschumacher (4) briefly reported the isolation of a derivative of the enzyme after incubating asparaginase in 50% dimethylsulfoxide with the asparagine analogue, 5-diazo-4-oxo-L-norvaline, an inhibitor of asparaginase; they presumed this was an intermediate in the enzymatic pathway. Asparaginase itself, however, did not react to form a stable intermediate.

By means of kinetic analysis, we were able to use the reactions of asparaginase involving hydroxylamine to differentiate between a mechanism which could proceed through a β-aspartyl enzyme intermediate and a mechanism which would not. Analysis of the hydrolytic reaction alone was not suitable for this purpose because the concentration of water cannot be varied. The results of our kinetic studies using hydroxylamine and exchange studies with 18O-labeled water were consistent with a mechanism for asparaginase II of E. coli which involves a β-aspartyl enzyme intermediate. We propose that this mechanism involves the three symmetrical reactions shown in Fig. 1.

EXPERIMENTAL PROCEDURE

Materials—Salt-free hydroxylamine was used for all experiments and was obtained from hydroxylamine sulfate after precipitation of the sulfate as the barium salt. It was kept at 0° and used on the day of preparation. We determined the concentration of hydroxylamine by titration with HCl. No significant differences were seen in experiments with the use of hydroxylamine-HCl neutralized with NaOH. Uniformly labeled 14C-asparagine was obtained from Amersham-Searle, Arlington Heights, Illinois, and was purified as described previously (5); H218O (low in deuterium) came from Bio-Rad, Richmond, California. β-Aspartohydroxamic acid was synthesized from asparagine (6) and was also obtained from Nutritional Biochemicals, Cleveland, Ohio.

Enzyme—Two preparations of asparaginase II of E. coli were used, Worthington, nominally 32 units per mg of protein, and Nutritional Biochemicals, nominally 318 units per mg. The latter enzyme preparation, therefore, is presumed to approach 100% purity (7).

Enzyme Determinations—Incubations were carried out at 37°. Hydrolysis of asparagine was assayed in a volume of 50 μl containing 0.2 mM 14C-asparagine (specific activity 5 mCi per mmole) and 0.01 mM Tris-acetate buffer. Separation of the radioactive aspartic acid formed in 10-μl samples was accomplished by high voltage electrophoresis at pH 4.7 as previously described (5).

One unit of asparaginase is that amount of enzyme which in 1
min catalyzed the formation of 1 μmole of aspartic acid at 37° and pH 7.

β-Aspartohydroxamic acid was measured colorimetrically by the method of Lipmann and Tuttle (1). The ferric complex of both the synthesized product and that obtained commercially had a molar extinction at 500 nm of $0.5 \times 10^6$. In order to follow the hydrolysis of β-aspartohydroxamic acid, 0.5-ml samples were pipetted into an equal volume of acidified FeCl₃ reagent and read at 500 nm, the absorption maximum. With reaction mixtures containing hydroxylamine in concentrations greater than 0.5 m, the color obtained in the presence of the hydroxamic acid was more intense and different spectrally from the amber seen with lower concentrations of hydroxylamine, being rosier with substantial absorbance between 510 and 550 nm. Formation of the rose-colored complex was seen only when the hydroxylamine was initially at a pH above its pK (about 6.2, Reference 8) even though the pH of the ferric complex was finally below 1. Since the formation of this complex was unrelated to the enzymatic reaction, its nature was not investigated further. If the hydroxylamine in the reaction mixture was lower than 0.5 m, this artificial color was not seen. With higher concentrations, the contribution of the artificial color was corrected for by determining the molar extinction of the hydroxamate in the presence of hydroxylamine.

We also detected and identified the β-aspartohydroxamic acid produced in incubation mixtures with ¹⁴C-asparagine by radioautography after high voltage electrophoresis (5). Electrophoresis at pH 4.7 for 30 min separated asparagine from aspartic acid and the hydroxamic acid; electrophoresis in the second dimension at pH 1.9 for 33 hours separated asparagine from the hydroxamic acid. The radioactive product was also identified by paper chromatography which separated the β- from the α-aspartohydroxamic acid (5).

Isoelectric focusing was carried out for 48 hours as previously described (5) with ampholytes of pH 4 to 6 obtained from LKB Instruments, Rockville, Maryland.

Determinations of ¹⁸O were performed by mass spectrometry by Airmont Analytic Service, Suffern, New York and by Dr. Michael Caplow, Yale University, after combustion of aspartic acid as described previously (9). Isotope contents for the initial rate of the exchange were calculated from at least four spectrographic records using the formula of Shain and Kirsch (10).

RESULTS

Formation of β-Aspartohydroxamic Acid—In the presence of hydroxylamine, asparaginase catalyzed the formation of β-aspartohydroxamate with either asparagine or aspartic acid. The initial rate of the hydroxylaminolysis of asparaginase was dependent upon the concentration of enzyme but was linear with time only when less than 10% of the substrate was converted (Fig. 2); the product disappeared if substantial concentrations of the hydroxamate accumulated (Fig. 2A), indicating that the hydroxamate was readily hydrolyzed by the enzyme (see below).

The dependence of the rate of hydroxylaminolysis on the concentration of hydroxylamine at pH 7 is shown in a double reciprocal plot (Fig. 3). The apparent activation of the enzyme at the higher hydroxylamine concentrations is artifactual and resulted from an effect of hydroxylamine on the color of the ferric complex (see "Experimental Procedure"). The $K_m$ for hydroxylamine was 40 μM. The $V_{max}$, which was obtained by extrapolation to the ordinate in Fig. 3, was 0.36 μmole per min per ml. For these determinations, we used 0.34 unit of asparaginase per ml. It is thus apparent that at pH 7 the enzyme can catalyze the hydroxylaminolysis and the hydrolysis of asparagine at similar rates.

Formation of β-aspartohydroxamate from aspartic acid at pH 7 was readily detected and proceeded at 10% of the rate of asparaginase hydrolysis (Fig. 4). Whereas the $K_m$ for asparagine in the asparaginase reaction was 5 μM (see Fig. 3A), the enzyme was fully saturated at pH 7 only by concentrations of aspartic acid greater than 10 mM.

Purified asparaginase II of E. coli has been shown to catalyze the hydrolysis of glutamine (11) and of β-cyanoalanine (12). Glutaminohydroxamate was formed at 5% of the rate of the hydroxylaminolysis of asparagine at pH 7. The rate of the endogenous glutaminase activity was also about 5% of the asparaginase activity (11). In incubations containing 24 mM β-cyano-
Enzymatic Mechanism of Asparaginase

Vol. 264, No. 1

Fig. 3. The dependence of initial rates of the hydroxylaminolysis of asparagine on the hydroxylamine concentration at pH 7.1. Incubations contained 0.05 M asparagine, 0.34 unit of asparaginase (Nutritional Biochemicals) per ml, 0.05 M Tris-acetate buffer (pH 7.1) and various concentrations of hydroxylamine.

Fig. 4. Formation of β-aspartohydroxamate from aspartic acid. Incubations contained 0.5 M hydroxylamine, 0.5 unit of enzyme (Nutritional Biochemicals), 0.1 M Tris-HCl (pH 7.2), and 5 mM asparagine (A) or 50 mM aspartic acid (B).

Fig. 5. Isoelectric focusing of asparaginase. Focusing was performed (see under "Experimental Procedure") for 48 hours in an ampholyte gradient between pH 4 and pH 6. Asparaginase (Worthington, 19 units) and 2 mg of crystalline bovine serum albumin were added to the column. Fractions of 4 ml were collected. pH (— —) was measured at 0° with a Radiometer pH meter standardized with cold pH 4 buffer. After making these measurements, we neutralized the fractions by the addition of 1 M potassium phosphate buffer, pH 7. Hydrolysis of asparagine (O—O) was assayed by conversion of [14C]-asparaginase to aspartate. Hydroxylaminolysis of asparaginase (Δ—Δ) was measured at pH 7.2 in 1 M Tris-HCl buffer, 0.5 M hydroxylamine, and 5 mM asparagine.

We assumed that the extinction coefficients of the ferric complexes of the hydroxamates formed were similar to that of β-aspartohydroxamic acid.

Asparaginase preparations of different purity produced similar results in all of the experiments with hydroxylamine. In order to show that both the hydroxylaminolysis and the hydrolysis of asparagine were catalyzed by the same enzyme, Worthington's partially purified enzyme was subjected to pH gradient electrophoresis (Fig. 5). The asparaginase activity banded in a single peak with an isoelectric point of 4.9, confirming an earlier determination of Campbell and Mashburn (11). The hydroxylaminolysis activity corresponded exactly in position to the asparaginase activity.

Hydrolysis of β-Aspartohydroxamic Acid—At pH 7 asparaginase catalyzed the hydrolysis of β-aspartohydroxamic acid at a maximal rate about 1.6 times that of the hydrolysis of asparagine. The $K_m$ for the hydroxamate was 3 mM (Fig. 6). This hydrolysis accounts for the disappearance of product seen in Fig. 2A.

pH-Dependence of Asparaginase-catalyzed Reactions with Hydroxylamine—Campbell et al. (13) have reported that the maximal rates of asparagine hydrolysis by L-asparaginase II of E. coli showed a broad optimum in the neutral region, but that the velocities did not diminish sharply at more acid values of pH. We have confirmed their results (Fig. 7). A similar dependency was observed with the hydrolysis of β-aspartohydroxamate and for the formation of β-aspartohydroxamate from aspartic acid. The hydroxylaminolysis of asparaginase, however, showed a different pattern of pH dependency with an optimum at pH 5.6. This
pH, in addition to being quite near the pK of hydroxylamine, is also the optimum for the nonenzymatic formation of hydroxamates from asparagine and glutamine (3) and from various other amides (14). Meister et al. (3) have shown that asparaginase from guinea pig serum also catalyzed the formation of β-aspartohydroxamic acid from asparagine with an optimal maximal velocity at about pH 6. Our results are similar to those of Meister et al. (3) except that the rates of hydroxylaminolysis catalyzed by asparaginase II of E. coli are comparable to or greater than the rates of asparagine hydrolysis at all values of pH.

Studies with 18O-Labeled Water—The formation of β-aspartohydroxamate from aspartic acid is an indication that asparaginase activates the β-carbonyl group of aspartate. We obtained further evidence for this interaction from the exchange of oxygen between 18O-labeled water and aspartic acid which, after 15 hours, approached the expected equilibrium value of 0.1 atom % excess (Table I). This incorporation required the enzyme but was not dependent upon the presence of added ammonia.

The incorporation of 16O into aspartic acid increased linearly with time (Fig. 8). The values obtained at the earlier times represent amounts too small to be measured accurately. The initial rate of this exchange reaction was $3.5 \times 10^{-4}$ atoms % per min. This rate was 12% of the maximal velocity of the hydrolysis of asparagine, which in turn was similar to that of the formation of the hydroxamate from aspartic acid.

Initial Velocity Studies with Hydroxylamine—Two possible kinetic models can be proposed for asparaginase catalysis (Mechanisms I and II). If we assume that the hydroxylaminolysis reaction proceeds through the same catalytic pathway as does the hydrolysis of asparagine, then we may use the reaction with hydroxylamine to distinguish between the two models.

In Mechanism I asparagine first adds to the enzyme, releasing ammonia and forming an aspartyl enzyme. This intermediate can react either with water to form aspartic acid or with hydroxylamine to form the hydroxamic acid. In Mechanism II asparagine is first bound to the enzyme. This complex may react with water to release aspartic acid and ammonia from the enzyme, or it may react with hydroxylamine to form the hydroxamic acid and ammonia. Mechanism I might involve an acyl intermediate whereas Mechanism II could not. Kinetically these two mechanisms are different (15).
Fig. 8. Initial rate of exchange between $^{18}$O-labeled water and aspartic acid catalyzed by asparaginase. Asparaginase (Nutritional Biochemicals, 0.75 unit) was incubated at 37°C in a volume of 0.6 ml in water containing 0.04 M aspartic acid brought to pH 7 with a small amount of 2 M NaOH, and bovine serum albumin at a final concentration of 0.1 mg per ml. Samples of 0.5 ml were removed at the indicated times. Protein was precipitated from the chilled samples by the addition of 0.5 ml of 10% trichloroacetic acid following the addition of 0.9 mg of bovine serum albumin as carrier. After centrifugation, trichloroacetic acid was extracted from the resulting supernatants with ether. Aspartic acid was dried by lyophilization followed by treatment in an Abderhalden apparatus for 1 hour. Aspartic acid from an incubation at 37°C for 30 min without enzyme contained 0.2303 atoms % excess $^{18}$O. The data presented was obtained after subtracting this background value. The $^{18}$O content of water was found to be 5.55 atoms % excess.

Fig. 9. Initial rate studies. A, plot of reciprocal initial velocities against reciprocal asparagine concentration. Incubations contained Worthington asparaginase and 0.05 M Tris-HCl buffer (pH 7.8) in a volume of 70 μl. The sum of $\gamma$-aspartohydroxamic acid and aspartic acid produced from $^{14}$C-asparagine was determined after high voltage electrophoresis at pH 1.9 (see under "Experimental Procedure"); the rate of production of both products (v) is given in micromoles per min per ml. No hydroxylamine was added for Curve 1. The concentrations for the other curves were: Curve 2, 0.12 M; Curve 3, 0.24 M; Curve 4, 0.48 M. B, vertical intercepts from A replotted against hydroxylamine concentrations.

Initial rate studies measuring formation of the hydroxamic acid and varying the concentrations of asparagine and hydroxylamine, however, cannot be used to distinguish the two mechanisms because water interferes as an alternate substrate. Nevertheless these studies were carried out and yielded an intersecting pattern as predicted by the rate equations for either mechanism.¹

In order to avoid the difficulty posed by the enormous concentration of water we might measure the initial rates of formation of ammonia, the common product of both hydroxylaminolysis and hydrolysis, with asparagine and hydroxylamine as variable substrates. We would predict from the rate equations that Mechanism I would give a parallel pattern, and Mechanism II, an intersecting pattern. This prediction, apparent from the mathematical analysis, was not intuitively obvious to us. The difference between the two mechanisms, however, is that in Mechanism II ammonia comes from two different forms of the enzyme, whereas in Mechanism I, ammonia comes from one form of the enzyme.

¹ Unpublished results.
enzyme. Since the formation of ammonia in small amounts is technically difficult to determine, especially in the presence of hydroxylamine, we have measured the formation of the sum of radioactive aspartic acid and $\beta$-aspartohydroxamate produced from $^{14}$C-asparagine. In Fig. 9A we have plotted the results of this experiment. The parallel pattern obtained indicated that hydroxylamine acts as an uncompetitive inhibitor of asparaginase and provides kinetic support for Mechanism I. The intercepts of these lines have been replotted as a function of the hydroxylamine concentration in Fig. 9B. A linear relationship was obtained; the apparent inhibition constant for hydroxylamine is about 0.3 M. From the rate equation for alternate substrate inhibition we would have expected that this plot should have been hyperbolic (16). Although our data appeared linear over the small range of hydroxylamine concentrations examined, the curve might become hyperbolic if studied over a larger concentration range.

### DISCUSSION

A plausible mechanism for the reactions catalyzed by asparaginase is shown in Fig. 1 and involves the formation of a $\beta$-aspartyl enzyme intermediate. In support of this mechanism we have demonstrated by initial rate studies (Fig. 9A) that product formation proceeded by a ping-pong mechanism. The present report may be the first application of this kind of kinetic analysis to a hydrolytic enzyme; the analysis might be made a general one for examining the mechanism of other hydrolytic transferases by the use of a variety of acceptors other than hydroxylamine.

The formation of $\beta$-aspartohydroxamate from aspartic acid can be interpreted as the product of the hydroxylaminolysis of an acyl enzyme intermediate. The fact that at pH 7 the initial rate of formation of the hydroxamate from aspartic acid (Reactions $-3$ and $+2$, Fig. 1) was the same as the rate of incorporation of $^{18}$O from water into aspartic acid (Reactions $-3$ and $+3$) argues strongly for a mechanism in which water and aspartic acid associate with the enzyme independently of ammonia (or of hydroxylamine). Of the two possible kinetic models (Mechanisms I and II), only Mechanism I predicts these results.

We have made the reasonable (but unproved) assumption that hydroxylamine in (Reaction 2) enters into the asparaginase reaction as an analogue of water (in Reaction 3) or ammonia (in Reaction 1). A possible indication that reactions with hydroxylamine proceed through the same catalytic pathway as water might be that the rates of reactions with hydroxylamine were quite similar to those which involve water. Rates for the various reactions catalyzed by asparaginase are listed in Table II. Moreover, hydroxylamine at moderate concentrations inhibited the hydrolysis of asparagine (Fig. 9). Although the type of inhibition is not ascertainable since water is not a variable substrate, this inhibition was consistent with the idea that hydroxylamine and water react with the enzyme at the same site.

Our mechanism predicts that Reaction 1, the acylation of the enzyme by asparagine, should be reversible. We have shown that both Reactions 2 and 3 are reversible. Attempts to detect the formation of asparagine from $^4$H-labeled aspartic acid and ammonia were unsuccessful. We probably failed because the specific radioactivity of the aspartic acid available was insufficient.

If our proposed mechanism is correct, each of the rates measured involves two reactions whose individual rates we have not been able to measure independently. The individual rates of Reaction 3 may be estimated, however, from the rates of $^4$O-labeled exchange ($v_{-4}$ and $v_{+4}$) and from the rate of formation of the hydroxamate from ammonia (Reactions $-3$) and $v_{+2}$). Thus $v_{+2}$ must be at least 10 times greater than $v_{+4}$. At pH 7 the rates of hydrolysis of asparagine ($v_{+2}$ and $v_{+4}$) and of $\beta$-aspartohydroxamate ($v_{+2}$ and $v_{+4}$) were similar to the rate of the hydroxylaminolysis of asparagine ($v_{+2}$ and $v_{+4}$) (Table II). A comparison of these rates of reaction is consistent with the notion that they occur by the same mechanism. Similar rates of reaction, however, do not necessarily indicate a similar reaction pathway. Thus we have not determined directly the rate-limiting step of any of these reactions. Furthermore, even if we assume that hydrolysis and hydroxylaminolysis proceed through the same catalytic pathway, we cannot take for granted that the rates of the individual component reactions are the same both in the presence and in the absence of hydroxylamine. In fact we have provided evidence which shows that the hydrolysis of asparagine is inhibited in the presence of hydroxylamine (Fig. 9).

The reactions of asparaginase II of *E. coli* involving hydroxylamine provide rapid and convenient assays for this clinically important enzyme. Either the hydroxylaminolysis of asparagine or the disappearance of $\beta$-aspartohydroxamate may be followed colorimetrically, and both procedures might be automated.

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