Raushel, F. M., & Cleland, W. W. (1977a) *Biochemistry 16*, 2169–2175.
Raushel, F. M., & Cleland, W. W. (1977b) *Biochemistry 16*, 2176–2181.
Rendina, A. R., & Cleland, W. W. (1981) *Anal. Biochem. 117*, 213–218.
Rendina, A. R., & Cleland, W. W. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1921.
Rendina, A. R., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry* (preceding paper in this issue).
Rose, I. A., O'Connell, E. L., Litwin, S., & Bar-Tana, J. (1974) *J. Biol. Chem. 249*, 5163–5168.
Shoham, M., & Steitz, T. A. (1980) *J. Mol. Biol. 140*, 1–14.
Thorner, J. W., & Paulus, H. (1973) *J. Biol. Chem. 248*, 3922–3932.
Uyeda, K. (1970) *J. Biol. Chem. 245*, 2268–2275.
Viola, R. E., & Cleland, W. W. (1978) *Biochemistry 17*, 4111–4117.
Viola, R. E., & Cleland, W. W. (1980) *Biochemistry 19*, 1861–1866.
Wurster, B., & Hess, B. (1975) *Methods Enzymol. 41*, 488–493.

Kinetic Mechanism and Location of Rate-Determining Steps for Aspartase from *Hafnia alvei*†

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ABSTRACT: Coupled spectrophotometric assays that monitor the formation of fumarate and ammonia in the direction of aspartate deamination and aspartate in the direction of fumarate amination were used to collect initial velocity data for the aspartase reaction. Data are consistent with rapid equilibrium ordered addition of Mg^{2+} prior to aspartate but completely random release of Mg^{2+}, NH_4^+, or fumarate. In addition to Mg^{2+}, Mn^{2+} can also be used as a divalent metal with V_{max} 80% and a $K_{aspartate}$ 3.5-fold lower than when Mg^{2+} is used. Monovalent cations such as Li^+, K^+, Cs^+, and Rb^+ are competitive vs. either aspartate or NH_4^+ but noncompetitive vs. fumarate. A primary deuterium isotope effect of about 1 on both V and $V/K_{aspartate}$ is obtained with (3*R*)-L-aspartate-*3-d*, while a primary ^{15}N isotope effect on $V/K_{aspartate}$ of 1.0239 ± 0.0014 is obtained in the direction of aspartate deamination. A secondary isotope effect on V of 1.13 ± 0.04 is obtained with L-aspartate-*2-d*. In addition, a secondary isotope effect of 0.81 ± 0.05 on V is obtained with fumarate-d_2, while a value of 1.18 ± 0.05 on V is obtained by using (2*S*,3*S*)-L-aspartate-*2,3*-d_2. These data are interpreted in terms of a two-step mechanism with an intermediate carbanion in which C–N bond cleavage limits the overall rate and the rate-limiting transition state is intermediate between the carbanion and fumarate.

Aspartase (EC 4.3.1.1) catalyzes the divalent metal dependent deamination of L-aspartate to yield fumarate and ammonia (Quastel & Woolf, 1926). The aspartase reaction was first demonstrated in bacteria by Harden (1901). Aspartase has been regarded as a catabolic enzyme in both bacteria and plants, but the reaction is reversible and favors aspartate formation with $\Delta G° = 3.2$ kcal/mol for aspartate deamination. The equilibrium constant for the aspartase reaction, measured directly at 25 °C by Bada & Miller (1968), is 5×10^{-3} M.

Nucleotides such as IMP, AMP, and GDP (to a lesser extent) and adenosine activate aspartase, while GTP and UTP inhibit (Williams & Scott, 1968). The nucleotide triphosphates increase $K_{aspartate}$ while the activators decrease $K_{aspartate}$; V_{max} is unaffected.

Gawron & Fondy (1959), using the data of Englard (1958) and Krasna (1958), showed that ammonia is added trans across the double bond of fumarate. The enzyme is specific for the amino acid substrate and fumarate, but NH_2OH can substitute for ammonia as a substrate (Emery, 1963). A variety of divalent metal ions will activate the reaction including Mg^{2+} and Mn^{2+} (Williams & Lartigue, 1967). The most complete study of the kinetic mechanism for aspartase was carried out by Dougherty et al. (1972), who suggested a uni-bi rapid equilibrium random mechanism. However, these studies neglected any role of the metal ion.

In this study, we present data on the overall kinetic mechanism for aspartase from *Hafnia alvei*, which includes the divalent metal ion as a pseudoreactant. In addition, through the use of primary and secondary kinetic isotope effects, it is suggested that the deamination of aspartate proceeds via a carbanion intermediate and that C–N bond cleavage limits the overall rate.

Materials and Methods

Chemicals and Enzymes. H. alvei aspartase (1 unit/mg), chicken liver malic enzyme, pig heart fumarase, bovine liver glutamate dehydrogenase, pig heart aspartate aminotransferase, and pig heart malate dehydrogenase were obtained

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from Sigma. Fumarate-d_2 (98 atom %) and $(^{15}NH_4)_2SO_4$ (95 atom %) were obtained from Merck and Co. L-Aspartate-*2-d* was prepared by using L-aspartate in D_2O with aspartate aminotransferase and was the generous gift of Dr. W. W. Cleland.

Preparation of (3R)-L-Aspartate-3-d. Fumarate (100 mM) and $(NH_4)_2SO_4$ (500 mM) were dissolved in 10 mL of D_2O (98 atom %) and titrated to pD 7.0 with KOD (98 atom %). The solution was rotary evaporated to near dryness with the subsequent addition of 10 mL of D_2O. The pD was maintained at 8.0. This procedure was repeated twice, and 50 mL of D_2O was added after the final evaporation. To this mixture was added solid $MgSO_4$ to a final concentration of 30 mM, and 50 units of aspartase was added to start the reaction. In order to monitor the time course for aspartate production, aliquots were taken from the reaction mixture, to these perchloric acid was added to quench the reaction, and the resulting solution was then titrated to pH 8.0. The amount of aspartate produced was determined from ΔOD_{340} by using 1 unit of aspartate aminotransferase, 1 unit of malate dehydrogenase, 10 mM 2-oxoglutarate, 0.2 mM DPNH,[1] and 100 mM Tris-HCl, pH 8.0. Preparation of deuterated aspartate was complete in 1 h. The reaction mixture was then boiled for 10 min and filtered to remove protein. The resultant filtrate was titrated to pH 10.5 with KOH and rotary evaporated to near dryness to remove ammonia. Then 10 mL of H_2O was added and the process repeated. This procedure was then repeated with an additional 10 mL of H_2O. The pH was monitored after each addition of H_2O to ensure that it remained at 10.5. This solution was then titrated to pH 8 with perchloric acid and allowed to stand for 1 h. The solution was then filtered to remove $KClO_4$ and the amounts of fumarate, malate, and aspartate were determined enzymatically. The malic enzyme reaction was used to detect the concentration of malate. The reaction mixture contained 1 unit of malic enzyme, 100 mM Tris-HCl, pH 8, 1 mM TPN, and 1 mM $MgSO_4$; the concentration of malate in the solution was 3% of the total aspartate plus malate plus fumarate. The assay conditions used for fumarate were the same as for malate, except 2 units of fumarase was added; the concentration of fumarate was 1.5% of the total. The final concentration of (3*R*)-L-aspartate-*3-d* was 235 ± 10 mM.

The above solution was further purified by using ion exchange on Dowex AG 1-X8 in the chloride form. The solution at pH 1.0 was applied to a 1 × 30 cm column of the ion-exchange resin, and the column was washed with 5 column volumes of water. The aspartate was then eluted with 2 N pyridine. Fractions containing aspartate were rotary evaporated to remove pyridine, and the thick concentrated solution was dried over P_2O_5. The 90-MHz ^1H NMR spectrum for (3*R*)-L-aspartate-*3-d* indicated the compound was at least 98 atom % D, while L-aspartate-*2-d* was at least 90 atom % D.

L-[^{15}N]Aspartate and (2*S*,3*S*)-L-aspartate-*2,3-*d_2 were prepared as above, except that H_2O replaced D_2O and $(^{15}NH_4)_2SO_4$ replaced $(NH_4)_2SO_4$ for the [^{15}N]aspartate preparation, while fumarate-d_2 replaced fumarate in the aspartate-d_2 preparation. Attempts were made to measure the ^{15}N isotope effect with both $^{15}NH_4^+$ and L-[^{15}N]aspartate, by direct comparison of initial velocities. However, all that could be stated was the ^{15}N isotope effect was smaller than 5%, since this is the largest isotope effect that could have been measured.

Solutions of aspartate were calibrated enzymatically with aspartate aminotransferase and malate dehydrogenase. The conditions were similar to those used for the coupled spectrophotometric assays described below, except no aspartase or Mg^{2+} was added to the reaction mixture and an amount of aspartate calculated to produce a 0.6–1.0 absorbance change was added.

The NH_4^+ solutions were calibrated by using glutamate dehydrogenase, while the fumarate solutions were calibrated by using the fumarase and malic enzyme couple with aspartase, Mg^{2+}, and NH_4^+ absent.

Enzyme Assays. Aspartase has been assayed continuously by employing the absorbance of fumarate at 240 nm (ϵ_{240} = 2255 M^{-1} cm^1) (Cook et al., 1980). Aspartase obtained commercially, however, is contaminated with fumarase, which eliminates monitoring A_{240} as a viable assay unless aspartase can be purified. Physical characterization of aspartase and fumarase indicates they are very similar in molecular weights. Aspartase has a M_r of 180 000 and is a tetramer (Williams & Lartigue, 1967), while fumarase has a M_r of 190 000 and is also a tetramer (Kanarek & Hill, 1964). In addition, aspartase is reported to have a p*I* of 4.8 (Ellfolk, 1956; Wilkson & Williams, 1961), while the contaminating fumarase has a p*I* of 5.0 from isoelectric focusing (obtained by Fred S. Gruman in this laboratory). Their activities can be separated by a variety of chromatographic techniques as well as starch gel electrophoresis (Wilkinson & Williams, 1961), but the yield is so poor that it is economically unfeasible. An affinity column has been synthesized by Chibata et al. (1974), in which aspartate is attached by its amino group to Sepharose via a six-carbon arm. This resin does not separate *H. alvei* aspartase activity from fumarase activity, even though it is reported to work for the *Escherichia coli* enzyme (Chibata et al., 1974).

Thus, in order to study this enzyme, coupled spectrophotometric assays have been developed that couple the production of aspartate, fumarate, or ammonia to an enzyme-catalyzed reaction(s) that produces or utilizes pyridine nucleotides. The production of fumarate is coupled to the fumarase and malic enzyme reactions so that the production of TPNH can be monitored at 340 nm. A typical assay in a 1-mL volume contained 46 units of fumarase, 5.4 units of malic enzyme, 100 mM Tris-HCl, pH 8.0, 1 mM TPN, 1 mM DTT,[1] 0.1 mM EDTA, 12 mM free Mg^{2+} (15 mM total), 1 mM free aspartate (4 mM total), and variable aspartase. Optimization of this and all coupled assay systems used was obtained by using the method of Cleland (1979a). A plot of velocity vs. aspartase is linear up to 0.002 unit/mL. An activity per milliliter of 1.8 units was obtained from the same stock solution that was used to check all coupled assays.

The production of ammonia was coupled to the glutamate dehydrogenase reaction and the disappearance of TPNH was monitored. A typical assay in a 1-mL volume contained 119 units of glutamate dehydrogenase in 50% glycerol, 100 mM Tris-HCl, pH 8, 50 mM 2-oxoglutarate, 0.2 mM TPNH, 1 mM DTT, 0.1 mM EDTA, 1 mM aspartate (4 mM total), 12 mM free $MgSO_4$ (15 mM total), and variable aspartase. A plot of velocity vs. aspartase concentration is linear up to 0.032 unit/mL. For the same stock solution used above, a value of 1.6 units/mL was obtained with this couple. Thus, the two coupled assays equally reflect the rate of aspartase.

The production of aspartate from fumarate and ammonia was coupled to the aspartate aminotransferase and malate dehydrogenase reactions, and the disappearance of DPNH was

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid.

monitored. Since contaminating fumarase is present, enough fumarase, 92 units, was added to bring fumarate and malate to equilibrium rapidly. The equilibrium constant for the fumarase reaction is 4 in the direction of malate production (Blanchard & Cleland, 1980); therefore, the fumarate added was 5 times the desired concentration. No inhibition of the aspartase reaction by malate was detected when compared to the same assay minus added fumarase. A typical assay in a 1-mL volume contained 92 units of fumarase, 39 units of aspartate aminotransferase, 26 units of malate dehydrogenase, 100 mM Tris-HCl, pH 8, 2 mM 2-oxoglutarate, 0.2 mM DPNH, 1 mM DTT, 0.1 mM EDTA, 1 mM free fumarate (2.2 mM total), 250 mM $(NH_4)_2SO_4$, 12 mM free $MgSO_4$ (13.2 mM total), and variable aspartase. A plot of velocity vs. aspartase concentration is linear up to approximately 0.04 unit/mL. In each of the cases in which a different coupled assay was used, care was taken to maintain the aspartase concentration within the limits of linearity of the assay.

Initial Velocity Studies. All data were collected by using a Gilford 2600 spectrophotometer and a Hewlett-Packard flatbed plotter to plot the time course and its derivative. The temperature was maintained at 25 °C with a circulating water bath with the capacity to heat and cool the thermospacers in the cell compartment. All reactions were carried out in a 1-mL cuvette with a 1-cm light path. All reactions were initiated by the addition of aspartase. Fumarate and aspartate concentrations were corrected for complexation with divalent metals by using the following dissociation constants obtained at 0.1 M ionic strength: Mg–aspartate, 4 mM; Mg–fumarate, 10 mM; Mn–aspartate, 0.2 mM (Dawson et al., 1971). The pH dependence of V/K_{NH_4} was obtained by maintaining Mg^{2+} (12 mM uncomplexed) and fumarate (2 mM uncomplexed) saturating and varying the NH_4^+ concentration. The coupled assay was checked at pH 6.0 and 10.0 to be sure aspartase was limiting. Buffers used at 100 mM concentration were Mes, pH 6.0, Hepes, pH 7.0, Tris-HCl, pH 8.0, Taps, pH 8.0, Ches, pH 9.0, and Caps, pH 10.0. Several of the assays were repeated at a given pH with one of the other buffers to be sure no inhibition or activation was obtained; none was detected. At the pH extremes, the concentrations of fumarate and Mg^{2+} were doubled in separate assays to be sure they were still saturating; no significant rate change was detected.

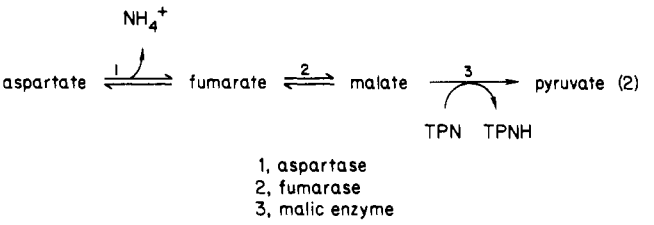
Determination of the ^{15}N Isotope Effect. The $^{15}(V/K)_{aspartate}$ isotope effects were determined by isotope-ratio mass spectral analysis of the NH_3 from complete-conversion and low-conversion (10–20% reaction) samples. The analysis of the complete-conversion sample gives the $^{15}N/^{14}N$ ratio in the initial aspartate, and the $^{15}N/^{14}N$ ratio of the low-conversion sample reflects the discrimination against the heavier isotope according to the equation

$$^{15}(V/K) = \frac{\log (1 - f)}{\log (1 - fR/R_0)} \quad (1)$$

where R_0 is the isotope ratio in the initial aspartate and R the isotope ratio of the product NH_3 at fractional conversion f.

For precise isotope-ratio analysis it is necessary to convert the NH_3 to N_2. This is accomplished by HOBr oxidation of the isolated and purified ammonia. The overall experimental protocol is described in detail below.

Since aspartase is reversible, determination of the kinetic isotope effect on the deamination of aspartate requires a method of continuous removal of fumarate to prevent the back-reaction. The use of fumarase and malic enzyme as coupling enzymes converts the fumarate to pyruvate and CO_2 (HCO_3^- at pH 7.9) according to the scheme



By use of an appropriate excess of fumarase and malic enzyme, the aspartase reaction was kept rate limiting and the fumarate concentration low.

The assay solution for the low-conversion reaction consisted of 50 mM aspartate, 10 mM TPN, and 5 mM glucose 6-phosphate in 100 mM Hepes buffer, pH 7.9 (titrated with NaOH), containing 60 mM $MgCl_2$. A glutamate dehydrogenase assay[2] of this solution indicated the NH_4^+ concentration was less than 50 μM. To each of eight 10.0-mL samples of the above solution were added 2 units of malic enzyme and 10 units of fumarase (both of which had been desalted on a G-10 column and assayed with glutamate dehydrogenase to ensure low NH_4^+ content). To one 10.0-mL sample of the assay solution were added 4 units of malic enzyme and 20 units of fumarase. All nine of these low-conversion samples were incubated with 0.25 unit of aspartase at 25 °C for up to 5 h. During this period 20-μL aliquots of the assay solution were removed and diluted to 1.0 mL, and the absorbance at 340 nm due to TPNH formation was measured to determine the extent of reaction. After a period of time appropriate for 13–19% reaction, 50 units of G6PDH (low in NH_4^+) was added to convert unreacted TPN to TPNH. After 10 min this solution was applied to a 10 × 1.7 cm column of AG MP-1 and eluted with 20 mM LiCl, pH 7.5. The fractions containing NH_4^+ were pooled and the glutamate dehydrogenase assay for NH_4^+ showed quantitative recovery. From the absorbance at 260 nm, less than 1 μmol of nucleotide was determined to be present in this pool. It is important to remove the nucleotide because, under the conditions required for steam distillation, a decomposition of nucleotide to yield ammonia occurs.

Six 10.0-mL complete-conversion samples were treated identically with that described above. Initial concentrations of 8.0 mM aspartate, 10.0 mM TPN, and 5 mM glucose 6-phosphate were used. Incubation was allowed to proceed for 8 h with 1 unit of aspartase, 4 units of malic enzyme, and 20 units of fumarase. After this period of time the absorbance at 340 nm was appropriate for 100% reaction of aspartate.

The ammonia from the low- and complete-conversion samples was isolated by steam distillation (Bremner, 1965). The acidic distillate was assayed for NH_4^+ and concentrated from 50 mL to approximately 1 mL by rotary evaporation at 45 °C and reduced pressure (vacuum pump). This solution was oxidized by using 1.0 mL of hypobromite (prepared by adding 60 mL of Br_2 to 300 mL of 17 M NaOH and diluting this 1:1 with 12 mM KI)[3] in a "Y"-tube (Bremner, 1965). The $^{15}N/^{14}N$ ratio of the resulting N_2 was measured with a Varian MAT 250 dual-inlet isotope-ratio mass spectrometer. The reproducibility of this oxidation (to 0.00002% of the $^{15}N/^{14}N$ isotope ratio) has been demonstrated.[4] In addition, the manipulations involved in the isolation of the NH_3 through steam

[2] A Hepes-buffered solution at pH 8.0 containing 30 mM 2-oxoglutarate, 0.3 mM TPNH, and 100 units of glutamate dehydrogenase was used for the spectrophotometric assay of NH_4^+.

[3] This mixture is filtered 48 h after preparation and stored at 4 °C in the dark.

[4] Unpublished results of P. M. Weiss.

distillation have been shown not to introduce any isotopic fractionation.

Data Processing. Reciprocal initial velocities were plotted vs. reciprocal substrate concentrations, and all plots were linear with the following exceptions. The aspartate saturation curve exhibits positive cooperativity, and both fumarate and NH_4^+ saturation curves exhibit negative cooperativity [in agreement with the results of Williams & Lartigue (1967)]. All data were fitted to appropriate rate equations by using the Fortran programs of Cleland (1979b). Data for the initial velocity patterns in the direction of aspartate deamination were fitted line by line to eq 3, but all replots were linear and fitted to

$$v = \frac{VA^2}{a + 2bA + A^2} \quad (3)$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (4)$$

$$v = \frac{VA}{K(1 + 1/K_{is}) + A} \quad (5)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (6)$$

$$v = \frac{VA}{K + A} \quad (7)$$

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (8)$$

$$\log y = \log\left(\frac{C}{1 + K_1/H}\right) \quad (9)$$

the equation for a straight line. In the case of the reverse reaction, concentrations of reactants were maintained below the concentrations that produced negative cooperativity. Data for initial velocity patterns in the direction of fumarate amination were fitted to eq 4. Data for inhibition patterns were fitted to eq 5 and 6. The saturation curve for Mn^{2+} was fitted to eq 7. Data obtained for isotope effects by direct comparison of initial velocities were fitted to eq 8. Data obtained for the pH dependence of V/K_{NH_4} were fitted to eq 9. In eq 3–8, V is the maximum velocity, and K is the Michaelis constant for the varied substrate. The subscripted K's in eq 4 indicate which substrate the K_m refers to, and K_{ia} is the kinetically determined dissociation constant for A. In eq 8, F_i is the fractional concentration of heavy atom label (for 95 atom % D, $F_i = 0.95$), while E_V and $E_{V/K}$ represent the isotope effect minus 1 on the respective parameter. In eq 9, y is the apparent V/K_{NH_4}, C is the pH-independent value of V/K_{NH_4}, K_1 is the dissociation constant for some group on enzyme or NH_4, and H is the hydrogen ion concentration. The nomenclature used for isotope effects is that of Northrop (1975) as modified by Cook & Cleland (1981).

Results

Initial Velocity Studies. When uncomplexed aspartate is varied at different fixed levels of uncomplexed Mg^{2+}, the initial velocity pattern shown in Figure 1 is obtained. Intersection on the ordinate is indicative of rapid equilibrium ordered addition of Mg^{2+} prior to aspartate. Kinetic parameters are listed in Table I. When Mg^{2+} is varied and aspartate is maintained at a fixed concentration, apparent complete substrate inhibition is observed. The reciprocal experiment results in a hyperbolic increase in rate with an increase in aspartate concentration. These data suggest that Mg^{2+} and aspartate and not the Mg–aspartate complex are reactants, and the data

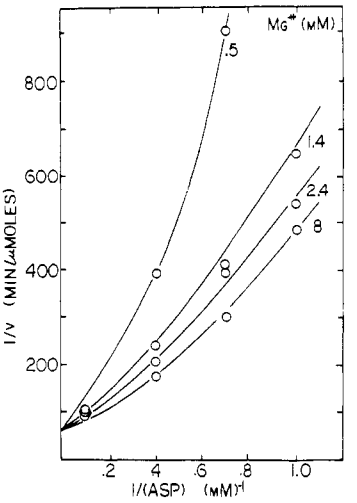


FIGURE 1: Initial velocity pattern for aspartate deamination at pH 8, 100 mM Tris-HCl. The Mg^{2+} and aspartate concentrations are corrected for the Mg–aspartate complex. Data were fitted line by line to eq 3.

Table I: Kinetic Parameters for Aspartase[a]

parameter	value ± SE
	Mg^{2+}
V_f (μmol/min)	0.0025 ± 0.0002
V_r (μmol/min)	0.0034 ± 0.0003
$K_{aspartate}$ (mM)	2.5 ± 0.4
K_{Mg}[b] (mM)	1.05 ± 0.15
K_{NH_4} (mM)	112 ± 25
$K_{fumarate}$ (mM)	0.2 ± 0.02
$K_{i\,Mg}$[c] (mM)	1.7 ± 0.02
$K_{i\,fum}$[d] (mM)	0.135 ± 0.031
$V/K_{asparate}$ (min^{-1})	0.001 ± 0.0003
V/K_{NH_4} (min^{-1})	(6.3 ± 2.5) × 10^{-5}
$V/K_{fumarate}$ (min^{-1})	0.019 ± 0.001
V/K_{mg} (min^{-1})	0.004 ± 0.0004
	Mn^{2+}
V_f (μmol/min)	0.002 ± 0.0002
$K_{i\,Mn}$[c] (mM)	0.012 ± 0.001
$K_{asparate}$ (mM)	0.7 ± 0.01
$V/K_{asparate}$ (min^{-1})	0.003 ± 0.0005

[a] Data were obtained by using the coupled enzyme assays discussed under Materials and Methods and fitted as discussed under Data Processing. [b] Obtained in the fumarate amination direction. [c] K_i is for dissociation from the E–M^{2+} complex. [d] K_i is for dissociation from the E–NH_4–fumarate complex.

are consistent with rapid equilibrium addition of Mg^{2+} prior to aspartate.[5] In addition, when the aspartate and Mg^{2+} initial velocity data are replotted as reciprocal velocity vs. reciprocal Mg–aspartate concentration, four distinct lines are observed rather than one line, indicating Mg^{2+} and aspartate bind separately and not as the chelate complex. In the direction of fumarate amination three initial velocity patterns were obtained by varying NH_4^+ at different fixed concentrations of fumarate and saturating Mg^{2+}, varying fumarate at different fixed levels of Mg^{2+} and saturating NH_4^+, and varying NH_4^+ at different fixed concentrations of Mg^{2+} and saturating fumarate. All patterns intersect to the left of the ordinate; kinetic parameters are listed in Table I.

It has previously been reported (Williams & Lartigue, 1967) that divalent metal ions other than Mg^{2+} will activate as-

[5] Even though the concentration of Mg–aspartate increases with increasing aspartate concentration when Mg^{2+} is fixed, as long as uncomplexed Mg^{2+} is at least stoichiometric to enzyme, aspartate at high concentrations will trap metal on enzyme and shift the equilibrium toward E–Mg–aspartate.

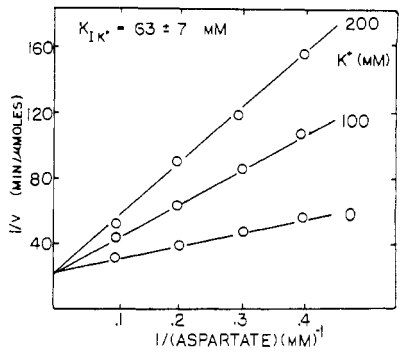


FIGURE 2: Competitive inhibition by K^+ vs. aspartate. The Mg^{2+} concentration was fixed at 12 mM. Data were fitted to eq 5.

partase. In order to test this, Mn^{2+} was substituted for Mg^{2+}. Mn^{2+} is used at 80% the maximum rate of Mg^{2+}. As seen in Table I, Mn^{2+} binds about 2 orders of magnitude tighter than Mg^{2+} and decreases the K_m for aspartate by a factor of 3.5. In addition, Zn^{2+} and Co^{2+} were tested but activated only to a slight extent, if at all, while Ca^{2+} gave no rate (probably as a result of its large ionic radius).

To determine whether NH_4^+ or NH_3 is the substrate for aspartase in the direction of fumarate amination, the saturation curve of NH_4^+ was obtained as a function of pH. The V/K_{NH_4} decreases above a pK of 9.30 ± 0.15.

Inhibition Studies. Inhibition patterns obtained using both products, fumarate and NH_4^+, were competitive vs. aspartate with K_i values of 0.13 ± 0.02 mM and 100 ± 15 mM, respectively. Furthermore, inhibition competitive against aspartate was observed by the monovalent cations Li^+ (K_i = 54 ± 3 mM), K^+ (K_i = 64 ± 9 mM), Cs^+ (K_i = 43 ± 14 mM), and Rb^+ (K_i = 45 ± 7 mM), with no inhibition by sodium.[6] An inhibition pattern for K^+ vs. aspartate is shown in Figure 2. A value of 63 ± 7 mM is obtained for the K_i. In addition to competitive inhibition by K^+ vs. aspartate, K^+ is also competitive vs. NH_4^+ with a K_i of 80 ± 10 mM but noncompetitive vs. fumarate with K_{is} of 80 ± 6 mM and a K_{ii} of 180 ± 13 mM.

Isotope Effects. Isotope effects were obtained on V and $V/K_{aspartate}$ by direct comparison of initial velocities with (3*R*)-L-aspartate-*3*-(*h*,*d*) as substrates. Isotope effects[7] are ^DV = 1.04 ± 0.004 and $^D(V/K_{aspartate})$ = 1.02 ± 0.012. The primary ^{15}N isotope effect was not different than 1 when attempts to measure by direct comparison of initial velocities were made. Thus, the $^{15}(V/K)_{aspartate}$ isotope effect was determined from comparison of $^{15}N/^{14}N$ ratios in the N_2 from the NH_3 of partial and complete-conversion reactions. These ratios are tabulated in Table II. The mean value for $^{15}(V/K)_{aspartate}$ is 1.0239 ± 0.0014.

Secondary deuterium isotope effects were obtained by using L-aspartate-*2*-*d*, (2*S*,3*S*)-L-aspartate-*2*,*3*-d_2 and fumarate-d_2. Direct comparison of initial velocities was used to obtain values for secondary isotope effects. As a result of curvature in the reciprocal plots, $^D(V/K)$ values were not defined, so ^DV values are reported. All reactants were prepared and purified by using ion-exchange chromatography, and thus it is not likely that the isotope effects are a result of a competitive inhibitor

[6] Na^+ does not inhibit significantly up to 200 mM concentration, but Li^+, which has a smaller ionic radius, does inhibit. This may be a result of Li^+ having a higher charge to mass ratio than Na^+ and, thus, binding with its primary hydration sphere. When this is taken into account, Li^+ is about the same size as K^+.

[7] All isotope effects were repeated between 3 and 6 times with similar results. The fumarate and fumarate-d_2 were recrystallized prior to use to eliminate the possibility of inhibitors present in either solution.

Table II: ^{15}N Isotope Effects at pH 7.9, 25 °C, for Aspartase

substrate	% reaction[a]	isotope ratios[b] (×10⁵) low conversion	100% conversion	$^{15}(V/K)$
aspartate	13.0	361.96	369.45	1.0222
	14.0	361.91	369.67	1.0231
	14.8	362.33	369.67	1.0220
	15.3	361.91	369.45	1.0227
	18.3	361.94	369.67	1.0237
	15.7	361.95	370.35	1.0253[c]
	16.9	362.11	370.38	1.0251
	17.0	362.10	370.35	1.0250
	17.2	361.95	370.41	1.0257

$^{15}(V/K)_{aspartate}$ = 1.0239 ± 0.0014

[a] Value for the low-conversion sample determined from both the UV change at 340 nm due to the production of TPNH from the malic enzyme reaction and the amount of NH_4^+ in the steam distillate. [b] Ratios were corrected to the tank standard of 370.00. [c] The last four determinations were obtained on a separate day by using new stock solutions and are completely independent.

in the reactant solution. By use of aspartate-*2*-*d* an isotope effect of 1.13 ± 0.04 was obtained for ^DV, while a value of 1.18 ± 0.05 for ^DV was obtained by using (2*S*,3*S*)-L-aspartate-*2*,*3*-d_2. By use of fumarate-d_2 a V_{max} isotope effect of 0.8 ± 0.05 was obtained. All deuterium isotope effects were repeated between 3 and 6 times with very similar results.

Discussion

Kinetic Mechanism. When reciprocal velocity is plotted vs. the reciprocal of the Mg–aspartate concentration, all points do not fall on a single curve. In addition, progressive inhibition is obtained when the Mg^{2+} concentration is increased and the aspartate concentration is fixed. Mg^{2+} and aspartate are most likely the combining species for aspartase, not the Mg–aspartate complex.

The initial velocity pattern observed when uncomplexed aspartate is varied at several fixed concentrations of uncomplexed Mg^{2+} intersects on the ordinate, indicating rapid equilibrium ordered addition of Mg^{2+} prior to aspartate. In agreement with this, no inhibition is obtained when Mg^{2+} is fixed at a single concentration and aspartate is increased. The reciprocal plots for aspartate are curved, indicative of positive cooperativity in agreement with Williams & Lartigue (1967). Inhibition by the products of the reaction, fumarate and NH_4^+, is competitive with aspartate, indicating that the products combine to the same enzyme form as aspartate, the E–Mg^{2+} complex. These results suggest that no significant concentrations of E–Mg–fumarate or E–Mg^{2+}–NH_4^+ accumulate in the steady state (if the complexes even form) and the off rates for both products are much faster than the catalytic rate.

In the direction of fumarate amination, three initial velocity patterns were obtained by saturating with one substrate and varying the other two. All three patterns intersected to the left of the ordinate. In the two cases where Mg^{2+} was varied (saturating NH_4^+ or saturating fumarate), the rate varies with Mg^{2+} concentration and patterns were not parallel, suggesting that the saturating substrate does not add between Mg^{2+} and the other varied substrate. In addition, since the rate varies when Mg^{2+} is varied at saturating concentrations of both of the other reactants, there must be randomness in the addition of Mg^{2+} and the varied reactant. From initial velocity data in the direction of aspartate deamination, it is known that the E–Mg^{2+} complex forms and from product inhibition data E–Mg–fumarate and E–Mg^{2+}–NH_4^+ also form.

In the direction of fumarate amination, it was determined that NH_4^+ does not add between Mg^{2+} and fumarate and that

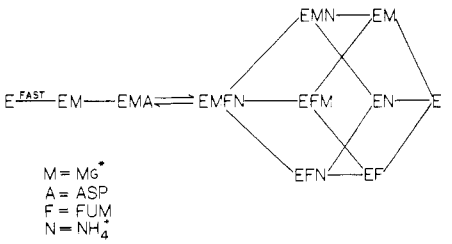


FIGURE 3: Kinetic mechanism for the aspartase reaction.

fumarate does not add between Mg^{2+} and NH_4^+ and that the rate varies at saturating NH_4^+ plus fumarate. Thus, the E–NH_4^+, E–fumarate, and E–fumarate–NH_4^+ complexes must also form.[8] Therefore, the mechanism in the direction of fumarate amination appears completely random as shown in Figure 3.[9] Inhibition by K^+ is competitive vs. NH_4^+ and noncompetitive vs. fumarate, which is expected for random addition of fumarate and NH_4^+. Dougherty et al. (1972) postulated a uni-bi rapid equilibrium random mechanism with the metal ion at a saturating concentration in agreement with the above. Since pure enzyme was used by Dougherty et al. (1972) and kinetic parameters in this study are in excellent agreement with those obtained by these authors, the contaminants in the enzyme preparation used for this study do not appear to affect the kinetic mechanism.

The enzyme β-methylaspartase, which catalyzes a very similar reaction, was postulated by Bright (1965) to have an identical kinetic mechanism in the amination direction, that is, completely random addition of Mg^{2+}, mesaconitate, and NH_4^+. However, random addition was also observed for Mg^{2+} and β-methylaspartate, unlike aspartase.

Since the K_m for aspartate decreased when Mn^{2+} was used as the divalent metal in place of Mg^{2+}, interaction of the metal with aspartate is suggested. This may be a coordination of the divalent metal with the β-carboxyl group. Since the dissociation constant of Mn–acetate is 63 mM while that for Mg–acetate is 316 mM (Dawson et al., 1971), and the ratio of these is about equal to the 3.5-fold decrease obtained in $K_{aspartate}$ when Mn^{2+} is substituted for Mg^{2+}, this supports the idea that the divalent metal is coordinated to only one of the carboxyl groups of aspartate. In contrast, as discussed under Materials and Methods, there is a 20-fold difference in the dissociation constants for Mg–aspartate and Mn–aspartate representing coordination to more than one functional group on aspartate. This reasoning is valid, however, only if the K_m for aspartate is equal to the K_d.

There appears to be a finite binding site for monovalent ions since competitive inhibition vs. aspartate and NH_4^+ is observed by ions such as Li^+, K^+, Cs^+, and Rb^+. This suggests that the α-amino group of aspartate, NH_4^+, and the other monovalent cations, once bound, are all either hydrogen bonded or ion paired to either a specific negatively charged residue on the enzyme or several electronegative atoms on the enzyme.

Interpretation of Isotope Effects. The primary deuterium isotope effect with (3*R*)-L-aspartate-*3-d* obtained on both V and V/K is very small, indicating that abstraction of this proton is not rate determining. However, a significant primary ^{15}N effect of 2.4% on V/K is obtained, indicative of at least partially rate determining carbon–nitrogen bond cleavage. Since the kinetic mechanism appears rapid equilibrium, the off rates for aspartate and all reactants are rapid with respect to catalysis and K_m is most likely equal to K_d. Thus, C–N bond cleavage probably limits the overall rate, and this step must be separated from proton abstraction from the 3*R* position. Either a two-step carbanion or carbonium ion mechanism for aspartase is consistent with these results. There may also be some rate limitation from slow conformation changes in the central complexes.

Secondary isotope effects have as a reference the equilibrium isotope effect, such that a value of 1 indicates a reactant-like transition state, and a value equal to $^DK_{eq}$ suggests a product-like transition state (Cook et al., 1980, 1981). This is only true if there are no finite commitment factors, e.g., rate-determining reactant or product release from the central complexes or slow conformational changes. Data for the determination of the kinetic mechanism rule out the former but do not rule out the latter of these as possibilities for finite commitment factors. The above criteria should apply to V and V/K effects in a rapid equilibrium mechanism with the additional assumption that there are no significant isotope effects on binding of reactants. This is certainly a reasonable assumption for the secondary deuterium effect.

A value of about 13% is obtained for the aspartate-*2-d* secondary isotope effect on V. The maximum possible secondary kinetic isotope effect would be a value of 1.28 (Cook et al., 1981), which is the product of an equilibrium secondary isotope effect of 1.12 for an sp^3 to sp^2 hybridization change and an equilibrium secondary isotope effect of 1.14 for deuterium on a carbon that has a nitrogen in place of a hydrogen. As with the primary effects, this secondary kinetic effect may reflect either the rate-determining transition state or finite internal commitment factors, that is, slow conformation changes in the central complexes. In view of the significant ^{15}N isotope effect, the most likely explanation is the former.

When fumarate-d_2 is used as the labeled reactant, a value of 0.69 should be obtained for the equilibrium isotope effect between fumarate and aspartate (Cleland, 1980). This $^DK_{eq}$ is a product of an sp^2 to sp^3 hybridization change at C-3, which has a $^DK_{eq}$ value of 0.89, and an sp^2 to sp^3 hybridization change at C-2 in which the deuterium is on carbon attached to a nitrogen as in aspartate; this $^DK_{eq}$ value is 0.77. A value of 0.81 is obtained for the kinetic isotope effect on V with fumarate-d_2. Since catalysis for this enzyme may occur in two steps, and values of 1.13 and 1.0 are obtained for the aspartate-*2-d* and (3*R*)-aspartate-*3-d* isotope effects, respectively, the simplest explanation for the fumarate-d_2 effect is that it reflects the hybridization change at C-2 only. The expected isotope effect for the case in which the transition state resembles aspartate is 0.77. The experimental value of 0.81 is slightly larger than this but more inverse than that predicted by the value of the aspartate-2-*d* effect (a value of 0.88 is predicted, 1.13/1.28). The standard errors on these values, however, are large enough so that these values are not significantly different.

The secondary effect on V obtained by using (2*S*,3*S*)-L-aspartate-*2,3-*d_2 should be the combined effects at the 2- and 3-positions of aspartate. The primary isotope effects indicate

[8] Metal will most likely add in rapid equilibrium independent of the enzyme complex to which it is absorbed. As a result, if it added only to free enzyme, saturating with reactants would effectively trap metal on enzyme no matter what its concentration and there would be no variation in rate. Since there is a variation in rate with varying Mg^{2+} concentration at saturating fumarate and ammonia, the E–fumarate–NH_4^+ complex must form. The lack of parallel patterns when either fumarate or NH_4^+ is saturating indicates that this ternary complex can be formed via addition of either fumarate or NH_4^+ first.

[9] It is necessary for the initial velocity data to adhere to the Haldane relationship. One of the Haldane relationships (Cleland, 1982) for the aspartase mechanism is $K_{eq} = (V_f/K_{aspartate})K_{i\ fumarate}K_{i\ Mg}/[(V_r/K_{NH_4})K_{i\ Mg}]$. The $K_{i\ Mg}$ value cancels and a value of 3.2×10^{-3} M is calculated for K_{eq}. This is in excellent agreement with the value of 5×10^{-3} M, which was determined directly by Bada & Miller (1968).

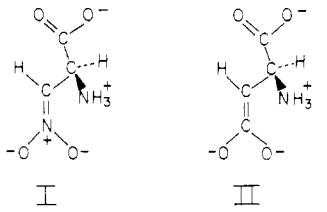


FIGURE 4: (I) Intermediate analogue for aspartase, 2-nitro-3-aminopropionate, and (II) proposed transition-state structure for the aspartase reaction.

a separation of the proton abstraction and C–N bond cleavage. If proton abstraction follows C–N bond cleavage (carbonium ion mechanism), only the C-2 effect (1.13) should be observed. If proton abstraction precedes C–N bond cleavage, however, the product of the effects at C-3 and C-2 will be observed (the product of 1.12 and 1.13). Considering the standard errors, the observed effect of 1.18 is consistent with either a large C-2 effect only or a full effect at C-3 multiplied by the C-2 effect. As will be discussed below, the most likely explanation is that C–N bond cleavage limits the overall rate and is preceded by hydrogen abstraction, with the transition state for C–N bond cleavage intermediate between aspartate and fumarate. Thus, it appears likely that the mechanism for aspartase is consistent with formation of a transient carbanion at C-3, similar to the reaction mechanism postulated by Blanchard & Cleland (1980) for fumarase, Bright (1964) for β-methylaspartase, and Schloss et al. (1980) for aconitase.

Porter & Bright (1980) have shown that 3-nitro-2-aminopropionate is an extremely good inhibitor of aspartase with a maximum K_m/K_i ratio of 220 at a pH where all of the compound exists as the *aci*-acid (Figure 4, I). Thus, the nitro compound is an intermediate analogue for aspartase, representing the stabilized *aci*-acid of aspartate as shown in Figure 4, II. This supports the tentative assignment made above from an analysis of primary and secondary isotope effects. In the absence of stabilization, the carbanion of compound II would be transient at best, but coordinated to a divalent metal ion it could be much more stable and in fact an intermediate. This, in fact, was also postulated by Bright (1964) for the metal-dependent β-methylaspartase reaction.

Further, Porter & Bright (1980) have shown that when compound I has a neutral amino group, it binds 8-fold better than when the amino group is protonated (K_m/K_i = 1630), and comparison of the neutral amino group of I with 3-nitropropionate indicates that without the amino group, binding is only 3-fold less. These observations could indicate that aspartase binds the 2– species of aspartate in which both carboxyls are ionized but the amino group is not. However, since linear competitive inhibition vs. aspartate and NH_4^+ is obtained by using a variety of monovalent cations, there is a binding site for the ionized α-amino group of aspartate. The overall charge of the suspected carbanion of aspartate is 2– as a result of the two negative charges of the β-carboxyl. The transition-state analogue, in order to have an overall charge of 2–, must have a neutral amino group. This may explain the 8-fold increase in affinity of the analogue with neutral amino group.

It is interesting to note that Mg^{2+} cannot be trapped on enzyme by fumarate[10] as it can with aspartate. Fumarate is bound 10 times more tightly than aspartate, and the K_m for fumarate is independent of Mg^{2+} concentration. In addition, the transition-state analogue 3-nitro-2-aminopropionate is bound very tightly and is planar at C-2 to C-3. Thus, it appears that enzyme preferentially binds planar molecules.

The V/K_{NH_4} decreases above a pK of about 9.3. This is most likely the pK for deprotonation of NH_4^+. Thus, it appears that the base that abstracts the 3R proton of aspartate in the deamination direction must be protonated in the direction of fumarate amination and NH_4^+ must be unprotonated (that is, these two functional groups have protonation states that are the reverse of the deamination direction). Chemically, this makes perfect sense since a positively charged α-amine is exactly what is required for elimination of NH_3 from aspartate, and NH_3 should be the attacking group in the amination direction. Thus, the concentration of the protonated base on enzyme (required to donate the 3R proton) and NH_3 will be optimum between the pK values for NH_4^+ and the enzyme base. The V/K_{NH_4} will decrease below the pK for the base[11] and above the pK for NH_4^+.

The reactions catalyzed by the ammonia lyases, aspartase, and β-methylaspartase are very similar to that catalyzed by fumarase. For the fumarase reaction some positively charged residue on enzyme is postulated to bind the β-carboxyl group and eventually stabilize the carbanion generated after deprotonation at C-3 (Blanchard & Cleland, 1980). In the case of aspartase, the divalent metal ion may play this role, by forming a bridge coordinating the β-carboxyl group and some enzyme residue. The fumarase reaction requires two bases (Brant et al., 1963), a carboxyl side chain that facilitates deprotonation at the 3R position and an imidazole that hydrogen bonds and eventually protonates the hydroxyl group to produce water as a product. In the case of aspartate, a base is still required to facilitate deprotonation at the 3R position, but no base is apparently required to protonate NH_3. Thus, only one base, that which protonates C-3, must be in the reverse protonation state for fumarate amination, with the attacking group being NH_3. For fumarate hydration both bases must be in the reverse protonation states since the one that protonated the leaving hydroxyl must deprotonate H_2O while the other must protonate the carbanion generated at C-3. Both aspartase and fumarase, however, require (1) the same stereochemistry for hydrogen abstraction at C-3 and (2) a stabilized *aci*-acid. The above mechanistic suggestions will serve as a hypothesis to test for the aspartase reaction mechanism.

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Registry No. Mg, 7439-95-4; NH_4, 14798-03-9; Li, 7439-93-2; K, 7440-09-7; Cs, 7440-46-2; Rb, 7440-17-7; Mn, 7439-96-5; ^{15}N, 14390-96-6; deuterium, 7782-39-0; L-aspartic acid, 56-84-8; fumaric acid, 110-17-8; aspartase, 9027-30-9.

References

Bada, J. L., & Miller, S. L. (1968) *Biochemistry 7*, 3403.

Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4506.

Brant, D. A., Barnett, L. A., & Alberty, R. A. (1963) *J. Am. Chem. Soc. 85*, 2204.

[10] Even when the fumarate is varied with the fumarate/NH_4^+ ratio constant at different Mg^{2+} concentrations, a pattern is obtained that intersects to the left of the ordinate. Thus, not even the presence of both reactants can trap Mg^{2+} on enzyme.

[11] This pK must be at least 5.0 since no decrease in V/K_{NH_4} was detected at pH 6.0.

Bremner, J. M. (1965) *Methods of Soil Analysis* (Black, C. A., Ed.) Part 2, pp 1256–1286, American Society of Agronomy, Inc., Madison, WI.
Bright, H. J. (1964) *J. Biol. Chem. 239*, 2307.
Bright, H. J. (1965) *J. Biol. Chem. 240*, 1198.
Chibata, I., Tosa, T., Sata, L., Sano, R., Yamamoto, K., & Matuo, Y. (1974a) *Methods Enzymol. 34*, 405.
Cleland, W. W. (1979a) *Anal. Biochem. 99*, 142.
Cleland, W. W. (1979b) *Methods Enzymol. 63*, 103.
Cleland, W. W. (1980) *Methods Enzymol. 64*, 94.
Cleland, W. W. (1982) *Methods Enzymol. 87*, 366.
Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 1790.
Cook, P. F., Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4845.
Cook, P. F., Oppenheimer, N. J., & Cleland, W. W. (1981) *Biochemistry 20*, 1817.
Dawson, R. M. C., Elliot, D. C., Elliot, W. H., & Jones, K. M. (1971) *Data for Biochemical Research*, p 430, Oxford Press, London, England.
Dougherty, T. B., Williams, V. R., & Younathan, E. S. (1972) *Biochemistry 11*, 2493.
Ellfolk, N. (1956) *Ann. Acad. Sci. Fenn., Ser. A2 No. 79.*
Emery, T. F. (1963) *Biochemistry 2*, 1041.
Englard, S. (1958) *J. Biol. Chem. 233*, 1003.
Gawron, O., & Fondy, T. P. (1959) *J. Am. Chem. Soc. 81*, 6333.
Harden, A. (1901) *J. Chem. Soc. 79*, 610.
Kanarek, L., & Hill, R. L. (1964) *J. Biol. Chem. 239*, 4207.
Krasna, A. I. (1958) *J. Biol. Chem. 233*, 1010.
Northrop, D. B. (1977) in *Isotope Effects in Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 122, University Park Press, Baltimore, MD.
Porter, D. J. T., & Bright, H. J. (1980) *J. Biol. Chem. 255*, 4772.
Quastel, J. H., & Woolf, B. (1926) *Biochem. J. 20*, 545.
Schloss, J. V., Porter, D. J. T., Bright, H. J., & Cleland, W. W. (1980) *Biochemistry 19*, 2358.
Wilkinson, J. S., & Williams, V. R. (1961) *Arch. Biochem. Biophys. 93*, 80.
Williams, V. R., & Lartigue, D. J. (1967) *J. Biol. Chem. 242*, 2973.
Williams, V. R., & Scott, R. M. (1968) *Biochem. Biophys. Res. Commun. 31*, 433.

Purification and Characterization of Three Forms of Collagenase from *Clostridium histolyticum*†

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ABSTRACT: Three collagenases from *Clostridium histolyticum*, designated C_1, C_2, and C_3, with apparent molecular weights of 96 000, 92 000, and 76 000 were purified. Peptide maps of the enzymes prepared by digestion with *Staphylococcus aureus* V-8 protease were found to be similar. Cleavage of native C_1 with α-chymotrypsin or V-8 protease yielded C_2 and C_3. This suggested that proteolysis of the M_r 96 000 collagenase may have occurred in vivo, producing the other two lower molecular weight enzymes. Previously prepared antiserum directed against a form of the bacterial enzyme similar by molecular weight and charge to collagenase C_3 and Fab′ fragments generated from this antiserum inhibited the collagenolytic activity. C_1, C_2, and C_3 were immunologically identical by Ouchterlony double diffusion, and C_3 was able to compete with C_1 for the antiserum binding site. The ability of each enzyme to bind to antiserum raised against the bacterial collagenase supported the hypothesis that these three proteins were closely related. Zinc analyses of C_1 and C_3 resulted in a value of 1.14 mol of zinc/mol of C_1 and 0.82 mol of zinc/mol of C_3. C_1 did not contain carbohydrate as measured by gas–liquid chromatography or periodic acid–Schiff staining.

Collagenases are defined as endopeptidases which cleave the triple helical region of the collagen molecule (Gross et al., 1974). They can be obtained from a variety of animal tissues (Eisen et al., 1970; Harper, 1980), and lower organisms such as fungi (Hurion et al., 1977) or bacteria (Strauch, 1974; Keil, 1979).

The literature contains many reports on the purification of collagenase from *Clostridium histolyticum*. Peterkofsky & Diegelmann (1971) used gel filtration to purify the enzyme. A number of investigators separated collagenolytic activity from contaminating proteases and noted multiple forms: Lwebuga-Mukasa et al. (1976) employed isoelectric focusing to obtain four species with different isoelectric points; Kono (1968) used DEAE-cellulose[1] chromatography to generate three enzymes with different specific activities; Harper et al. (1965) separated two species of different molecular weight on DEAE-cellulose.

The purpose of this study was to determine if the multiple forms of the clostridial collagenases were derived from one polypeptide and could be generated by proteolysis. Rabbit antiserum directed against one form of the collagenase was used to elucidate the immunological similarity of three purified collagenases. The collagenases were analyzed for amino acid composition and zinc and carbohydrate content.

Materials and Methods

The source of crude *C. histolyticum* collagenase, class IV (lots 48B019, 48K181, and 40D208) or class III (lots 47D261

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[1] Abbreviations: DEAE, diethylaminoethyl; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; TPCK, tosylphenylalanine chloromethyl ketone, Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin.

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