Schachman, H. K. (1959), Ultracentrifugation in Biochemistry New York, N. Y., Academic, p 82.
Sillen, L. G., and Martell, A. E. (1964), Stability Constants of Metal-Ion Complexes, London, The Chemical Society, Special Publication No. 17, p 411.
Spahr, P. F., and Edsall, J. T. (1964), *J. Biol. Chem. 239*, 850.
Stern, J. R., and Hegre, C. S. (1966), *Nature (London) 212*, 1611.
Wise, E. M., Jr., and Ball, E. G. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 1255.
Young, J. W., Shrago, E., and Lardy, H. A. (1964), *Biochemistry 3*, 1687.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

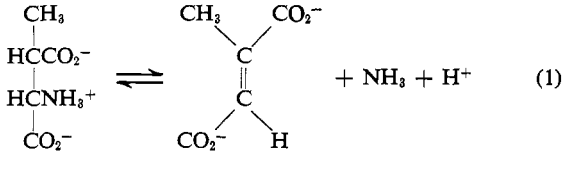
Magnetic Resonance and Kinetic Studies of the Activation of β-Methylaspartase by Manganese*

Gary A. Fields† and Harold J. Bright‡

ABSTRACT: Electron paramagnetic resonance studies of the interaction of Mn^{2+} with β-methylaspartase, which requires both monovalent and divalent metal cations for catalysis, show that the enzyme molecule has two Mn^{2+} sites. The Mn^{2+} sites act independently and the dissociation constant, K_s, for EMn^{2+} is 9×10^{-7} M. The binding of Mn^{2+} depends on an ionization with a pK_a value of 7.4. At pH 5.1, the binding of β-methylaspartate to EMn^{2+} tightens the binding of Mn^{2+} to the enzyme. Analysis of the affinity of Mn^{2+} for the enzyme as a function of the concentration of β-methylaspartate shows that the data are consistent only with a random-order addition of β-methylaspartate and Mn^{2+} to the enzyme. The values of the four dissociation constants for the random-order mechanism obtained with Mn^{2+} and β-methylaspartate from the electron paramagnetic resonance experiments (in the absence of K^+) are in excellent agreement with the corresponding values determined from steady-state turnover experiments in the presence of 0.15 M K^+.

This result, together with the fact that the K_m for K^+ is independent of the concentrations of β-methylaspartate and divalent metal activator at a given pH value, suggests that the binding of β-methylaspartate and divalent metal activator to the enzyme is independent of K^+. This is verified directly with Mn^{2+} in the case of K_s. However, the binding of Mn^{2+} to E–mesaconate is enhanced by K^+. Measurements of the effect of enzyme-bound Mn^{2+} on the longitudinal relaxation rate of water protons show that EMn^{2+} and K^+EMn^{2+} enhance the relaxation rate about 14-fold. The enhancement factor for EMn^{2+}–β-methylaspartate and EMn^{2+}–mesaconate is about 10-fold, and is 4-fold for K^+EMn^{2+}–mesaconate. These results are consistent with, but do not prove, a coordination structure in which Mn^{2+} acts as a bridge between substrate and enzyme.

β-Methylaspartase (*threo*-3-methyl-L-aspartate ammonia-lyase, EC 4.3.1.2) from *Clostridium tetanomorphum*, which requires both a monovalent and a divalent metal cation for reactivity, catalyzes the reversible conversion of *threo*-β-methyl-L-aspartate into mesaconate and ammonia (Barker *et al.*, 1959) according to eq 1. The enzyme has a molecular weight of 100,000 g/mole over a wide range of experimental conditions (Hsiang and Bright, 1967a) and can be dissociated in Gd·HCl[1] into subunits having a molecular weight of 50,000 g/mole (Hsiang, 1967; Hsiang and Bright, 1967b; Hsiang and Bright, 1969). The twenty cysteine residues in the molecule react with *p*MB at very different rates, all catalytic activity being lost after the first two cysteine residues have reacted with the mercurial (Hsiang and Bright, 1967a).



The existence and properties of an enzyme-catalyzed β-deuterium-exchange reaction with solvent protons (Bright *et al.*, 1964; Bright, 1964) led to the formulation of a mechanism which involves the formation of an enzyme-bound β-carbanion intermediate from which ammonia eliminates or is displaced in the rate-determining step of the overall reaction. Indirect evidence indicating that the sulfhydryl might be the base responsible for β-proton extraction from the substrate has been obtained both from studies of the β-deuterium-exchange reaction (Bright, 1964) and from photo-

* From the Department of Biochemistry, School of Medicine, University of Pennsylvania 19104. *Received March 19, 1970.* Financial assistance for this project by the National Institutes of Health, U. S. Public Health Service (Grant GM 11040), is gratefully acknowledged.

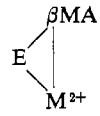
† Postdoctoral Trainee of the U. S. Public Health Service (Training Grant HD 0130). Present address: Department of Obstetrics and Gynecology, Hospital of the University of Pennsylvania, Philadelphia, Pa. 19104.

‡ Research Career Development Awardee 5-KO3-GM-34960, U. S. Public Health Service; to whom to address correspondence.

[1] The abbreviations used are: Gd·HCl, guanidine hydrochloride; E, active site of β-methylaspartase; βMA, *threo*-β-methyl-L-aspartate; MES, mesaconate; M^{2+}, divalent metal; *p*MB, *p*-mercuribenzoate.

oxidation experiments with β-substituted aspartic acids (Williams and Libano, 1966). It is fairly clear that the two essential sulfhydryl groups do not function as ligands for the divalent metal activator (Bright, 1965).

Systematic steady-state kinetic studies of the interaction of the enzyme with Mg^{2+} and Co^{2+} (Bright, 1965) and subsequently, with seven divalent metal activators and two divalent metal inhibitors (Bright, 1967) showed that βMA and M^{2+} add in a random-order, rapid-equilibrium fashion to the enzyme. In all cases βMA and M^{2+} were bound much more firmly to EM^{2+} and E–βMA, respectively, than to E. In addition, both the order of stability of the various binary, ternary, and quaternary complexes and the variation of the turnover number, k_{cat}, obtained with the various divalent metal ions tested, could be rationalized in terms of the cyclic bridge structure in which M^{2+} interacts with the β-carboxylate



group of βMA, thereby activating the β-proton for removal by a base at the active site and stabilizing the β-carbanion thus formed.

In view of the indirect nature of steady-state kinetic analysis, we considered it worthwhile to reinvestigate the mechanism of divalent metal activation of β-methylaspartase by more direct methods and compare the results so obtained with those from the steady-state kinetic experiments. We have used the electron paramagnetic resonance and pulsed nuclear magnetic resonance techniques which have been successfully applied by Cohn, Mildvan, and their coworkers (Cohn, 1963; Mildvan, 1970; Mildvan and Cohn, 1970) to a wide variety of Mn^{2+}-activated enzymes. We find two Mn^{2+} sites per molecule of β-methylaspartase and establish that the addition of βMA and Mn^{2+} to the enzyme is indeed a random-order, rapid-equilibrium process. The results of the pulsed nuclear magnetic resonance measurements are consistent with, but do not prove, a bridge function for M^{2+} in catalysis.

Experimental Section

Materials. Deionized water obtained from the passage of tap water over a mixed-bed resin (Continental Co., Philadelphia, Pa.) was further treated by passage over a Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) column. After the addition of 5×10^{-7} M EDTA, this water was used for all reagents and for final cleaning of glassware. No paramagnetic impurities could be detected in water prepared in this fashion.

β-Methylaspartase, which was electrophoretically pure and had a specific activity of 260 units/mg, was prepared as described previously (Hsiang and Bright, 1967a). Since such preparations are known to be contaminated with divalent metal activators and inhibitors (Bright, 1965), the enzyme (20 mg/ml in 0.5 M tetramethylammonium chloride) was incubated for 48 hr at 4° with 10^{-3} M EDTA. This solution was then concentrated to a minimal volume at 4° in a Schleicher & Schuell collodion bag apparatus. The enzyme was dissolved in 10 ml of 0.5 M tetramethylammonium chloride and 5-ml portions of this solution were treated as follows. After concentration to a minimal volume, the enzyme solution was mixed with 20 ml of 0.5 M tetramethylammonium chloride and then concentrated again. This procedure was repeated seven times. The enzyme (20 mg/ml) was stored as a stock solution in 0.5 M tetramethylammonium chloride at 4° and either used directly (for the magnetic resonance experiments) or diluted appropriately in 0.5 M tetramethylammonium chloride (for the kinetic experiments). Enzyme concentration was determined spectrophotometrically, after dilution of the stock solution about 200-fold in 0.05 M Tris-acetate (pH 7.0) using an ϵ_{279} value of 5.63×10^4 M^{-1} cm^{-1} (Hsiang and Bright, 1967a). We subsequently found that the slow loss of activity of stock enzyme solutions can be prevented by the addition of 10^{-3} M dithiothreitol.[2] Enzyme solutions to which dithiothreitol had been added were prepared for experiments (including the spectrophotometric determination of enzyme concentration) by passage over a 0.9×6.0 cm Sephadex G-25 (Pharmacia, Piscataway, N. J.) column equilibrated with the appropriate buffer solution. Although it was shown that dithiothreitol has a negligible affinity for Mn^{2+}, the oxidized product 4,5-dihydroxy-*o*-dithiane can interfere substantially with absorbance measurements at 279 mμ.

Tetramethylammonium chloride (Eastman, Rochester, N. Y.), Tris base, potassium chloride (Fisher, Philadelphia, Pa.), and mesaconic acid (H. M. Chemical Co., Santa Monica, Calif.) were recrystallized from water containing 10^{-5} M EDTA. *threo*-β-Methyl-L-aspartate was prepared by an established method (Barker and Smyth, 1960) and was also recrystallized from 10^{-5} M EDTA. Solutions of the reagents described above, including enzyme, contained insignificant paramagnetic impurities as determined by electron paramagnetic resonance and proton relaxation rate measurements.

Reagent grade manganous chloride was obtained from Fisher, Philadelphia, Pa., while standard manganous chloride solutions were obtained from Hartman-Leddon Co., Philadelphia, Pa.

Methods. Measurements of free Mn^{2+}. Concentrations of free Mn^{2+} were determined directly from the peak to peak heights of electron paramagnetic resonance spectra obtained on a Varian E-3 spectrometer with the solution temperature maintained at 25°. This is possible because complexes of Mn^{2+} have extremely broad electron paramagnetic resonance spectra (Cohn and Townsend, 1954); in our case, the amplitudes of the electron paramagnetic resonance spectra of EMn^{2+} and of βMA–Mn^{2+} could not be measured, indicating that they must be less than 5% of that of free Mn^{2+}. The sample (0.05 ml) was contained in a quartz capillary tube (1-mm i.d.) which was sealed at the bottom with paraffin wax and then placed in a regular electron paramagnetic

[2] The value of *n* (the number of Mn^{2+} sites per 100,000 g of protein) determined by the electron paramagnetic resonance method became as low as 1.3 after storage of the enzyme for several months. This effect paralleled a decrease in specific activity. The experiments shown in Figures 3 and 4 were carried out with enzyme which had been stored for four months and give *n* values varying from 1.33 to 1.54. We could show that in the presence of 4 mM β-methylaspartate (see Figure 3) or 4 mM mesaconate and 0.15 M KCl (see Figure 4), *n* values of between 1.8 and 2.1 could be obtained with freshly prepared enzyme, or with aged enzyme that had been treated with 10^{-3} M dithiothreitol.

resonance sample tube. Measurements were performed in the following sequence: (1) standard $MnCl_2$ solution, containing 0.3 M tetramethylammonium chloride; (2) free Mn^{2+} concentration in solution containing all reagents of interest except enzyme; and (3) free Mn^{2+} in complete solution, including enzyme.

Ionic strength in all experiments was maintained at 0.3 by the addition of the inert salt tetramethylammonium chloride.

MEASUREMENTS OF PROTON RELAXATION RATES OF WATER. Proton relaxation rate measurements were initially made at 25 Mcycles/sec by the pulsed nuclear magnetic resonance method in the Johnson Foundation, University of Pennsylvania, with the kind cooperation of Drs. Mildred Cohn and Albert Mildvan. Later pulsed nuclear magnetic resonance measurements were determined on an apparatus constructed by Mr. James Simmons which was operated at 14.45 Mcycles/sec and which utilized the magnet of the Varian E-3 spectrometer. Ionic strength was maintained at 0.3 with tetramethylammonium chloride and measurements were carried out at 25° on 0.1-ml solutions.

The observed enhancement, ϵ^*, of the paramagnetic contribution to the longitudinal relaxation rate of water protons (or proton relaxation rate) due to complexed Mn^{2+} is defined (Eisinger *et al.*, 1962) by eq 2, where T_1 and $T_{1(0)}$

$$\epsilon^* = \frac{\frac{1}{T_1^*} - \frac{1}{T_{1(0)}^*}}{\frac{1}{T_1} - \frac{1}{T_{1(0)}}} = \frac{\frac{1}{T_{1p}^*}}{\frac{1}{T_{1p(0)}}} \qquad (2)$$

are the observed longitudinal relaxation times for buffer solutions with and without Mn^{2+}, respectively, while T_1^* and $T_{1(0)}^*$ are the same parameters measured in the presence of a complexing agent for Mn^{2+}. The observed enhancement ϵ^*, is a weighted average of that due to all forms of Mn^{2+} (Mildvan and Cohn, 1963) according to eq 3, where X_i

$$\epsilon^* = \Sigma X_i \epsilon_i \qquad (3)$$

is the fraction of the ith Mn^{2+}-containing species and ϵ_i is the enhancement of the ith species. Neither βMA nor MES complex Mn^{2+} significantly under the experimental conditions used, and the enhancement values for the various Mn^{2+}-containing enzyme complexes were evaluated from ϵ^* and the concentration of free Mn^{2+} as determined by the electron paramagnetic resonance method.

KINETIC MEASUREMENTS. Rates of mesaconate formation were measured at 230 mμ (ϵ_{230} 5.59 × 10^3 M^{-1} cm^{-1}) on a Cary 15 spectrophotometer equipped with the 0–0.1-absorbance slide-wire. Solutions (1.0 ml) were maintained at 25° in quartz cuvets of 1.0-cm path length. The spectrophotometer slit width was maintained at 0.7 mm by means of the dynode and sensitivity controls. Total ionic strength was maintained at 0.3 with tetramethylammonium chloride and all titratable reagents were adjusted to the desired pH before use with Tris base of HCl.

Results

Enzyme Stability and Purity. In the past (Bright, 1965) we have experienced difficulty with divalent metal ion contamination of β-methylaspartase. In order to judge the efficiency of the exhaustive treatment of the enzyme with EDTA as described in the Experimental Section, contamination of the enzyme by divalent (and monovalent) metal activators was determined by rate measurements using the high concentrations of enzyme necessary for electron paramagnetic resonance and proton relaxation rate measurements. At pH 5.1, with saturating βMA (2.5 × 10^{-3} M) and 4.33 × 10^{-5} M enzyme, there was less than 0.1% conversion of βMA/min in the absence of added Mn^{2+} and/or K^+. Under the same conditions, but at pH 7.0 there was less than 1% conversion of βMA/min in the absence of added Mn^{2+} and/or K^+. Similarly, in the conversion of MES and ammonia into βMA at pH 5.1 and 7.0, there was essentially a complete dependence on added Mn^{2+} under similar conditions. Since ammonium also acts very efficiently as a monovalent activator of the enzyme, the dependence of the reverse reaction on added K^+ could not be tested under these conditions. These results indicate that in the electron paramagnetic resonance and proton relaxation rate measurements, which could be performed within a few minutes after adding enzyme to the reaction mixture lacking at least one essential substrate or K^+, negligible catalysis of the overall reaction had occurred.

We also established that the enzyme retained full activity for 120 min under the conditions used in the electron paramagnetic resonance and proton relaxation rate experiments.

Electron Paramagnetic Resonance Studies of the Interaction of Mn^{2+} with Enzyme. The interaction of Mn^{2+} with β-methylaspartase was studied by measuring the concentration of free Mn^{2+} in solutions of Mn^{2+} and enzyme, in the presence and absence of 0.15 M KCl, with enzyme concentrations in the range from 4.3 × 10^{-6} to 8.7 × 10^{-5} M. The results from several experiments at pH 5.1 are plotted in Figure 1 as the ratio of the total concentration of enzyme to the concentration of bound Mn^{2+} *vs.* the reciprocal of the concentration of free Mn^{2+}. Considering that the measurements shown in Figure 1 were performed with different enzyme concentrations (and two different enzyme preparations) over a period of several weeks, the fit of the data to a straight line is considered to be satisfactory.[2] The interaction of Mn^{2+} with n identical and noninteracting sites on the enzyme molecule is plotted according to eq 4 (Hughes and Klotz, 1956).

$$\frac{[E_T]}{[Mn_b^{2+}]} = \frac{K_{a,app}}{n[Mn_f^{2+}]} + \frac{1}{n} \qquad (4)$$

The value of n determined from the reciprocal of the ordinate intercept[2] is 1.7 in the absence of K^+ and 2.2 in the presence of 0.15 M K^+.

The value of $K_{a,app}$, the dissociation constant governing the interaction of Mn^{2+} with the Mn^{2+} sites at pH 5.1, is obtained from the reciprocal of the abscissa intercept of Figure 1 and is found to be 6.6 × 10^{-5} M in the absence of K^+ and 8.7 × 10^{-5} M in the presence of 0.15 M K^+. These values agree very well with the value of $K_{a,app}$ = 6.1 × 10^{-5} M determined for Mn^{2+} kinetically under identical conditions (see below) and also with the value of 3.2 × 10^{-5} M determined kinetically at a lower ionic strength of 0.135 (Bright, 1967).

The pH dependence of $K_{a,app}$ was studied by titration

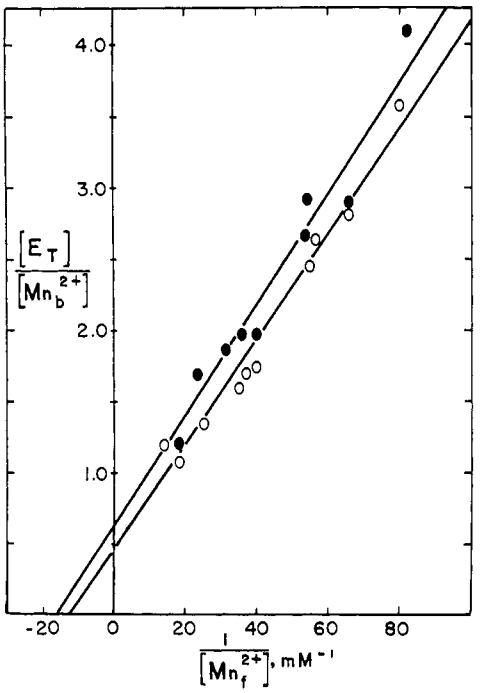


FIGURE 1: Titration of enzyme with Mn^{2+} at pH 5.1 and 25° in the absence (●) and presence (○) of K^+ by the electron paramagnetic resonance method. The electron paramagnetic resonance data are plotted according to eq 4 in the text giving $K_{a,app}$ as the reciprocal of the abscissa intercept and the number of Mn^{2+} bound per mole of enzyme as the reciprocal of the ordinate intercept. The lines represent least-squares fits to the measurements performed in the presence and absence of K^+. The conditions for these experiments were: Tris-acetate, (pH 5.1, 0.05 M); tetramethylammonium chloride, 0.25 M (●) or 0.10 M (○); KCl, none (●) or 0.15 M (○); enzyme concentration ranged from 2.2×10^{-5} to 8.7×10^{-5} M. Total $MnCl_2$ was varied between 10^{-5} and 10^{-4} M. Other details are given in Methods.

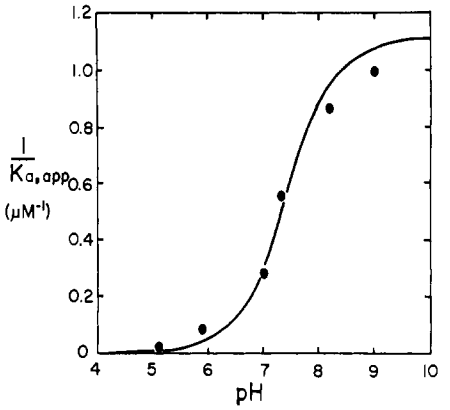


FIGURE 2: The pH dependence of $K_{a,app}$ at 25° as measured by the electron paramagnetic resonance method. The enzyme was titrated with Mn^{2+} and the data were analyzed as shown in Figure 1. The conditions for these experiments were: Tris-acetate, 0.05 M, pH values as indicated; tetramethylammonium chloride, 0.25 M. Enzyme concentrations were adjusted to compensate for the value of $K_{a,app}$, being 4.3×10^{-5} M for pH 5.1 and 5.9, 2.2×10^{-5} M for pH 7.0 and 4.3×10^{-6} M for pH 7.3, 8.2, and 9.0. The curve is calculated and corresponds to the titration of a group with $pK_a = 7.4$ and a limiting value for $K_a = 9 \times 10^{-7}$ M.

of the enzyme with Mn^{2+} and the results are presented in Figure 2. At the higher pH values, the affinity of the enzyme for Mn^{2+} becomes very great, and in order to avoid the condition $[E_T] \gg K_{a,app}$, which gives sharp Mn^{2+} titration end points corresponding to n but no information concerning $K_{a,app}$, substantially lower enzyme concentrations were used. Satisfactory spectra of free Mn^{2+} at concentrations as low as 10^{-6} M were obtained. Above pH 8.5, the rate of hydrolysis of free Mn^{2+} becomes significant, and no measurements were attempted in solutions more basic than pH 9.0. The limiting value of $K_{a,app}$, namely K_a, is approximately 9×10^{-7} M, which indicates that the enzyme behaves effectively as a metalloprotein at the higher pH values. The pK_{app} value of ligands on the enzyme for which Mn^{2+} and H^+ compete is 7.4. The pH dependence of $K_{a,app}$ demonstrated here was also qualitatively evident previously (Bright, 1967) in the results of kinetic studies. The computed number of Mn^{2+} sites per mole of enzyme[2] ranged between 1.7 and 2.0 throughout the entire pH range of Figure 2.

Experiments were also carried out in which Mn^{2+} was titrated with enzyme. When 8.7×10^{-6} M Mn^{2+} was titrated by successive additions of enzyme at pH 7.0, the measurements of free Mn^{2+} corresponded well with values calculated from $K_a = 9 \times 10^{-7}$ M and $pK_a = 7.4$ (see Figure 2). The addition of 10^{-3} M $ZnCl_2$ at the termination of the titration (when 1.85×10^{-5} M enzyme had been added and 94% of the Mn^{2+} was bound to the enzyme) caused the release of all the bound Mn^{2+}. The affinities of Zn^{2+} and Mn^{2+} for the enzyme are very similar, as judged by kinetic analysis (Bright, 1967).

Electron Paramagnetic Resonance Studies of the Interactions of Substrates and Mn^{2+} with the Enzyme. As discussed previously (Bright, 1967), the interpretation of data relating to the interactions of β-methylaspartase with Mn^{2+} and substrates, whether the experiments are kinetic or static, is considerably facilitated at pH 5.1 because at that pH neither βMA nor MES should bind a significant fraction of the divalent metal ion. Systematic electron paramagnetic resonance control experiments (see Experimental Section) indeed showed that at all concentrations of βMA, MES, and Mn^{2+} tested at pH 5.1 there was a negligible decrease in the concentration of free Mn^{2+} attributable to complex formation. However, the addition of βMA in experiments otherwise identical with those shown in Figure 1 caused a considerable decrease in the concentration of free Mn^{2+}. The enhancement of Mn^{2+} binding by the enzyme as a function of the concentration of βMA is shown in Figure 3. This result was anticipated from the results of steady-state kinetic studies of divalent metal activation of the enzyme (Bright, 1965, 1967) and is due to the fact that the affinities of βMA and Mn^{2+} for EMn^{2+} and E-βMA, respectively, are considerably greater than their affinities for E. The n values of less than 2 evident in Figures 3 and 4 almost certainly result from deterioration of the enzyme during prolonged storage.[2]

As in the case of βMA, the addition of MES to solutions of enzyme equilibrated with Mn^{2+} at pH 5.1, caused a considerable decrease in the concentration of free Mn^{2+} as shown in Figure 4. This must again be due to a mutual enhancement of the affinities of MES and Mn^{2+} for the enzyme. In this reaction direction, it was possible to investigate the additional effect of K^+ on the ternary complex

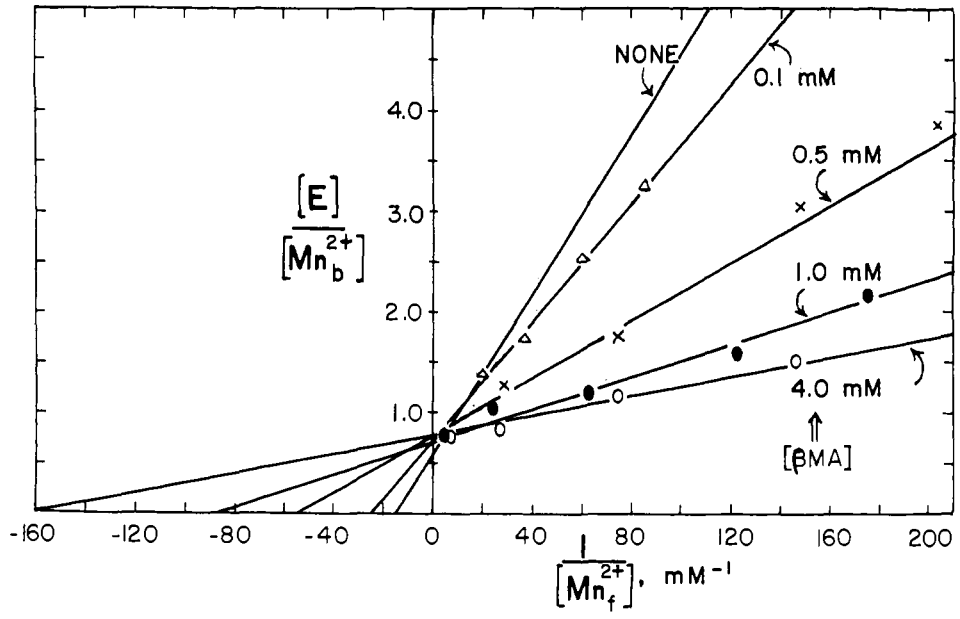


FIGURE 3: The effect of β-methylaspartate (Tris salt) on the binding of Mn^{2+} by the enzyme at pH 5.1 and 25° as determined by the electron paramagnetic resonance method. The concentration of β-methylaspartate is indicated by each line, and the line corresponding to no β-methylaspartate is that shown in Figure 1 with no K^+ present. Otherwise, conditions were identical to those given for Figure 1.

EMn^{2+}–MES in the absence of the second substrate, ammonia. As Figure 4 shows, the addition of K^+ (at a concentration of 1.5 times its K_m value in the forward reaction direction) to solutions of β-methylaspartase equilibrated with MES and Mn^{2+} at pH 5.1 increases the affinity of the enzyme for Mn^{2+}. Although we do not show the data, this Mn^{2+}-tightening effect shows the same K^+ dependence as the enhancement data obtained with K^+EMn^{2+}–MES (see Figure 5), with a half-maximal effect occurring at about 0.02 M K^+.

Proton Relaxation Rate Studies of the Interaction of Mn^{2+} with the Enzyme. The proton relaxation rate enhancement value for EMn^{2+} was found to be 13.0 at pH 5.1 and 14.6 at pH 7.0 in the presence of 0.25 M tetramethylammonium chloride. In the presence of 0.15 M K^+ and 0.1 M tetramethylammonium chloride, the enhancement values changed insignificantly, being 12.8 at pH 5.1 and 14.3 at pH 7.0. The experimental conditions and results of the proton relaxation rate experiments are summarized in Table I.

Proton Relaxation Rate Studies of the Interactions of Substrates and Mn^{2+} with the Enzyme. When a saturating concentration (as judged from the kinetic experiments) of βMA was added to EMn^{2+} at pH 5.1 and 25°, the proton relaxation rate enhancement value decreased from 13.0 to 10.9. A similar decrease of the enhancement, namely, from 14.6 to 9.2, was observed on adding a saturating concentration of βMA to EMn^{2+} at pH 7.0. Increasing the concentration of βMA from 4 to 10 mM did not change the enhance-

TABLE I: Summary of Proton Relaxation Rate Enhancement Values at 25° and Ionic Strength 0.3 for Various Complexes of the Enzyme.[a]

	EMn^{2+}	K^+-EMn^{2+}	EMn^{2+}-βMA	EMn^{2+}-MES	K^+-EMn^{2+}-MES
pH 5.1	13.0	12.8[b]	10.9[c]	9.0[c]	4.2[b]
pH 7.0	14.6	14.3[b]	9.2[c]	10.7[c]	4.2[b]

[a] The values are averages of duplicate measurements with each of two enzyme preparations, and the average error did not exceed ±8%. [b] In the presence of 0.15 M KCl (see Figure 5). [c] Maximum effect, in the presence of 4×10^{-3} M βMA or MES.

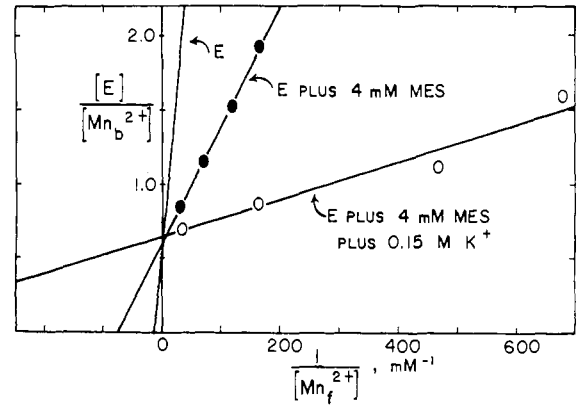


FIGURE 4: The effect of mesaconate (Tris salt) and K^+ on the binding of Mn^{2+} by the enzyme at pH 5.1 and 25°, as determined by the electron paramagnetic resonance method. The line representing the titration of enzyme alone is that shown in Figure 1 with no K^+ present. The concentrations of mesaconate and K^+ are indicated by the respective lines. Otherwise, conditions were identical with those given for Figure 1.

TABLE II: Comparison of Apparent Dissociation Constants at 25°, pH 5.1, and Ionic Strength 0.3 as Determined from Kinetic Experiments with 0.15 M K^+ and Electron Paramagnetic Resonance Experiments with No K^+.

Method	K_a (M)	K_a' (M)	K_s (M)	K_s' (M)
Kinetic	6.1×10^{-5}	2.0×10^{-6}	5.5×10^{-3}	1.7×10^{-4}
Electron Paramagnetic Resonance	6.5×10^{-5}	4.4×10^{-6}	2.0×10^{-3}	1.4×10^{-4}

ment at either pH. The experiments were performed, of necessity, in the absence of K^+.

The addition of saturating concentrations of MES to EMn^{2+} in the absence of K^+ caused reductions in the proton relaxation rate enhancement values which were similar to those caused by βMA, namely, from 13.0 to 9.0 at pH 5.1 and from 14.6 to 10.7 at pH 7.0. Increasing the concentration of MES from 4 to 10 mM did not change the enhancement at either pH. However, the addition of K^+ to EMn^{2+}–MES caused the enhancement to fall to a limiting value of 4.2 at both pH 5.1 and pH 7.0, as shown in Figure 5. It will be noted that the concentration of K^+ giving half-maximal decrease in the enhancement value is about 0.02 M at both pH 5.1 and pH 7.0, which is the same K^+ concentration necessary to half-titrate the Mn^{2+}-tightening effect seen in Figure 4 at pH 5.1. Although the K_m for K^+ activation in the forward reaction direction is also 0.02 M at pH 7.0, it is increased to 0.1 M at pH 5.1.

These enhancement results are also summarized in Table I.

Steady-State Kinetic Analysis of the Interactions of Mn^{2+} and β-Methylaspartate with the Enzyme. Since the conditions used for the present electron paramagnetic resonance and proton relaxation rate experiments were somewhat different with respect to ionic strength than those used in the steady-state kinetic investigations of Mn^{2+} activation reported previously (Bright, 1967), we considered it worthwhile to repeat the steady-state kinetics of Mn^{2+} activation of β-methylaspartase in the forward reaction direction. The results of these studies were interpreted in terms of a random-order, rapid-equilibrium addition of βMA and Mn^{2+} to the enzyme (see Discussion) and are summarized in Table II.

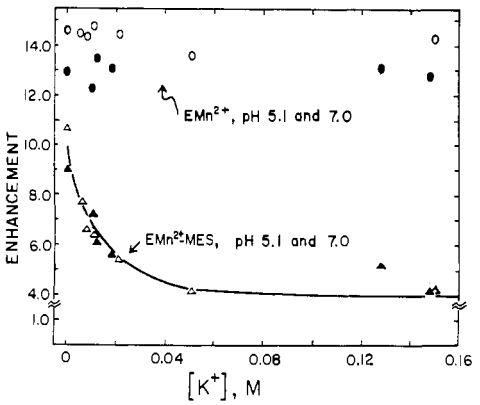


FIGURE 5: Titration of the EMn^{2+}–MES and EMn^{2+} complexes with K^+ at 25° according to the proton relaxation rate method. The enhancement of the proton relaxation rate of water due to enzyme-bound Mn^{2+} is plotted in the case of EMn^{2+}–MES at pH 5.1 (▲) and pH 7.0 (△), and in the case of EMn^{2+} at pH 5.1 (●) and pH 7.0 (○). The conditions for these experiments were: Tris-acetate, pH 5.1 or 7.0, 0.05 M; total $MnCl_2$, 4×10^{-5} M; Tris-mesaconate (lower curve), 4×10^{-4} M; tetramethylammonium chloride, 0.25 M; enzyme, 4.3×10^{-5} M. The concentrations of free Mn^{2+} were determined by the electron paramagnetic resonance method.

Discussion

The electron paramagnetic resonance measurements of the interaction of Mn^{2+} with β-methylaspartase in the pH range from 5.1 to 9.1 (comprising six independent graphical evaluations of the type shown in Figure 1) yield values of between 1.7 and 2.0 for the number of divalent metal activator sites per enzyme molecule, the average of these values being 1.9. In the presence of 0.15 M K^+ (Figure 1), 2.2 sites/molecule are found. The low values determined in the presence of βMA and MES (Figures 3 and 4) are almost certainly due to deterioration during storage of the enzyme preparations used in these particular experiments.[2] Taken together, these results strongly suggest that the β-methylaspartase molecule has two divalent metal activator sites. This conclusion, together with the fact that catalytic activity is completely lost after two cysteine residues have been reacted with *p*MB (Hsiang and Bright, 1967a) provides strong evidence that the β-methylaspartase molecule possesses two active sites. Since Gd·HCl splits the native enzyme (100,000 g/mole) into two subunits of molecular weight 50,000 g/mole (Hsiang, 1967; Hsiang and Bright, 1967b), it seems probable that each of the two subunits within the native enzyme contains a single active site. It is of interest that no evidence for site–site interactions of any kind has been obtained in these or previous studies (Bright, 1965, 1967).

By the following arguments, we show that the results of the electron paramagnetic resonance measurements of the interactions of Mn^{2+} and βMA with the enzyme at pH 5.1 in the absence of K^+ (Figure 3) are consistent only with a random order of addition. We discount the possibility that the ternary complex EMn^{2+}–βMA can be formed to any significant extent *via* the species βMA–Mn^{2+} because computation and direct electron paramagnetic resonance measurements show that βMA–Mn^{2+} accounts for a negligible fraction of the total amino acid present under the conditions employed and because kinetic and other considerations argue against the involvement of this complex as a substrate for the enzyme. This point has been discussed in previous publications (Bright, 1965, 1967) and we reemphasize it by pointing out that the concentration of βMA–Mn^{2+} at pH 5.1 (computed from the values for the α-ammo-

nium pK_a and the stability constant for βMA–Mn^{2+}), would require a rate of combination of βMA–Mn^{2+} with E which exceeds the diffusion controlled limit for such molecules in order to account for the observed turnover number of the enzyme. Since Mn^{2+} binds 10^3 times less well to MES than to βMA, similar considerations rule out MES–Mn^{2+} as an intermediate in the reverse reaction. The general scheme for the addition of Mn^{2+} and βMA to the enzyme is seen in Scheme I. We shall assume $[\beta MA]_{free} \approx [\beta MA]_{total}$, a condition which was clearly valid for all of the experiments shown in Figure 3 except for the line labeled 0.1 mM, where the concentration of βMA was only 2.5 times greater than the concentration of active sites. There are three possibilities to consider.

1. Ordered Addition: Substrate Followed by Mn^{2+}. This mechanism consists of the bottom half of the loop in Scheme I and can only be regarded as a possibility if the EMn^{2+} complex demonstrated by the experiments shown in Figure 1 is considered not to be involved in catalysis. Since the number of Mn^{2+} sites per enzyme molecule is in agreement with other evidence (Hsiang and Bright, 1967a) indicating that there are two active sites per molecule, and, more importantly, since the dissociation constant and other properties of the EMn^{2+} complex revealed by the electron paramagnetic resonance measurements are in excellent agreement with the results of kinetic experiments (see below), it seems most likely that the EMn^{2+} complex detected by the electron paramagnetic resonance measurements is identical with the EMn^{2+} complex involved in catalysis. On these grounds, therefore, the obligatory addition of substrate to the enzyme, followed by Mn^{2+}, is ruled out. We reached the same conclusion on the basis of kinetic experiments (Bright, 1965).

2. Ordered Addition: Mn^{2+} Followed by Substrate. This mechanism consists of the top half of the loop in Scheme I. It can be shown for this mechanism that

$$\frac{1}{[Mn_f^{2+}]_{\beta MA}} = \frac{[\beta MA]}{K_a K_s'} + \frac{1}{K_a} \qquad (5)$$

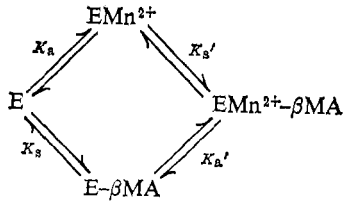
where $1/[Mn_f^{2+}]_{\beta MA}$ is the abscissa intercept in Figure 3 corresponding to a particular concentration of βMA. A plot of $1/[Mn_f^{2+}]_{\beta MA}$ *vs.* [βMA] should, therefore, be linear if this mechanism is correct. In fact, the plot is nonlinear (see inset of Figure 6), and we may conclude that the obligatory addition of Mn^{2+} to the enzyme, followed by βMA, is not supported by the data. This conclusion was also reached on the basis of kinetic experiments (Bright, 1965).

3. Random-Order Addition of Mn^{2+} and Substrate. This mechanism comprises the complete loop of Scheme I and is described by

$$\frac{1}{[Mn_f^{2+}]_{\beta MA}} = \frac{K_s}{[\beta MA]}\left(\frac{1}{K_a} - \frac{1}{[Mn_f^{2+}]_{\beta MA}}\right) + \frac{1}{K_a'} \qquad (6)$$

where $1/[Mn_f^{2+}]_{\beta MA}$ again represents the abscissa intercept in Figure 3 corresponding to a particular βMA concentration. Figure 6 shows that the data fit a straight line when plotted according to eq 6. We therefore conclude that the electron paramagnetic resonance measurements are satisfied by a random-order addition of βMA and Mn^{2+} to the enzyme, as shown in Scheme I. The values of K_a, K_s, K_a', and K_s'

SCHEME I



(the last being computed from the relationship $K_a K_s' = K_s K_a'$) determined by the electron paramagnetic resonance method of pH 5.1 are given in Table II. Because of ionizations of the enzyme, such as that shown in Figure 2 in the case of EMn^{2+}, these are apparent dissociation constants.

We originally deduced that βMA and Mn^{2+} add to the enzyme in a random-order, rapid-equilibrium fashion (Bright, 1965) from the analysis of steady-state turnover data obtained with Mg^{2+} and Co^{2+} as activators and from the protection afforded by βMA against inhibition of the enzyme by *p*MB in the absence of Mg^{2+}. Subsequently (Bright, 1967), we described the binding and kinetic parameters for nine different M^{2+}, including Mn^{2+}, as deduced from the random-order, rapid-equilibrium rate equation (eq 7). Attention was given

$$\frac{[E_T]}{v} = \frac{1}{k_{cat}}\left[1 + \frac{K_a'}{[M^{2+}]} + \frac{K_s'}{[\beta MA]} + \frac{K_a K_s'}{[M^{2+}][\beta MA]}\right] \qquad (7)$$

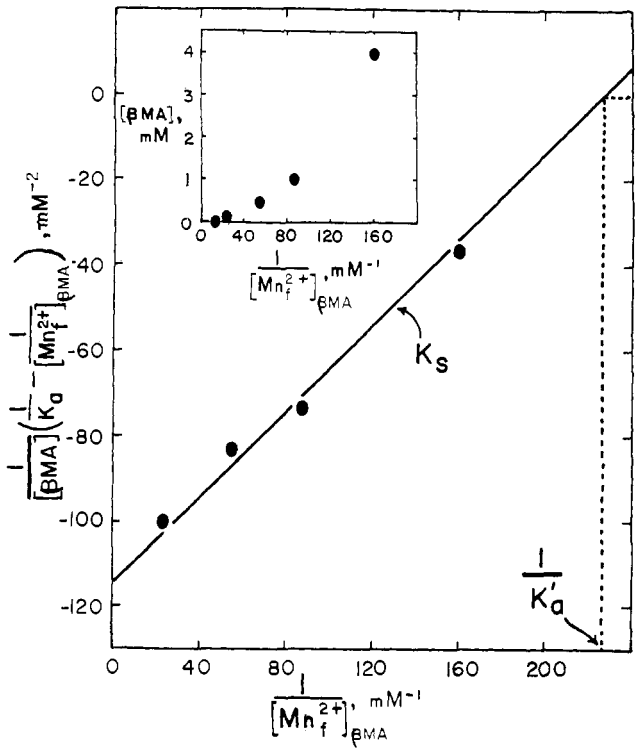


FIGURE 6: Plot of data of Figure 3 according to eq 5 in text, which corresponds to random-order addition of Mn^{2+} and β-methylaspartate to the enzyme. Inset shows plot of data of Figure 3 according to eq 4 in text, which corresponds to ordered addition of Mn^{2+} followed by β-methylaspartate.

to the problem of monovalent cation activation (K^+ was used then, as here), since it was recognized that if the various binary and ternary enzyme complexes were to bind K^+ with significantly different affinities, then the βMA and M^{2+} kinetics would be complicated at less than saturating levels of K^+. We found, however, that at a given pH the K_m values of βMA and Mg^{2+} were unaffected by K^+, and, likewise, that the K_m value for K^+ was unaffected by βMA and Mg^{2+}. We have repeated these experiments with Mn^{2+}, and have obtained identical results, the K_m values for K^+ being 0.1 and 0.02 M at pH 5.1 and 7.0, respectively. The invariance of the K_m value for K^+ over a wide range of concentrations of βMA and M^{2+} indicates that the K_m for K^+ represents a dissociation constant. Furthermore, judging from the kinetics of the forward reaction, K^+ acts solely to convert the enzyme from a catalytically inactive state into an active state, and has no effect upon the binding of βMA or M^{2+}. This conclusion is directly substantiated by the absence of a significant effect of K^+ (or NH_4^+) on the binding of Mn^{2+} by the enzyme (Figure 1). Consequently, the excellent quantitative agreement between the values of K_a, K_s, K_a', and K_s' determined in kinetic experiments with 10^{-8}–10^{-7} M enzyme and 0.15 M K^+, and the values determined in electron paramagnetic resonance experiments with 10^{-5}–10^{-4} M enzyme and no K^+, is taken to prove that the mechanism of M^{2+} activation of β-methylaspartase is accurately represented by the random-order formulation of Scheme I. Furthermore, since this agreement is only obtained when the rapid-equilibrium condition is imposed in the kinetic analysis, it must be concluded that the rates of dissociation of βMA and M^{2+} from the binary and ternary complexes are considerably greater than k_{cat}, the maximum turnover number.

The finding that K^+E–MES has a greater affinity than E–MES for Mn^{2+} at pH 5.1 (Figure 4) was somewhat surprising, since, as discussed above, the turnover kinetics indicate that the species K^+E–βMA has the same affinity for Mn^{2+} as does E–βMA at both pH 5.1 and 7.0.

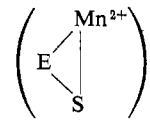
The possibility was considered that the activation of the enzyme requires the interaction of two K^+ ions with the enzyme, the first having a dissociation constant of 0.02 M (and representing the process reflected by the electron paramagnetic resonance and proton relaxation rate data in Figures 4 and 5, respectively), the second having a larger dissociation constant of about 0.1 M corresponding to the kinetically determined K_m value and involving no change in Mn^{2+} affinity or proton relaxation rate enhancement. It would then be expected that the K^+ activation kinetics in the forward reaction direction, if carried out over a very wide range of K^+ concentration, might reveal nonhyperbolic behavior. However, the response of initial velocity to K^+ was accurately hyperbolic over the entire range from 0.001 to 0.2 M. It should be noted that, in contrast to the results at pH 5.1, the K^+ dependence of the proton relaxation rate properties of K^+EMn^{2+}MES and of the initial velocity in the forward direction are identical at pH 7.0.

In the case of a paramagnetic species such as $Mn(H_2O)_6^{2+}$, the paramagnetic contribution ($1/T_{1p}$) to the observed longitudinal relaxation rates of solvent protons is given (Luz and Meiboom, 1964) by eq 8, where p is the ratio of the number of protons in the first coordination sphere of Mn^{2+} in chemical equilibrium with the solvent, to the total number

$$\frac{1}{T_{1p}} = \frac{p}{\tau_M + T_{1M}} \tag{8}$$

of solvent protons, τ_M is the lifetime of a water molecule in the coordination sphere, and T_{1M} is the relaxation time of a water proton in the coordination sphere of Mn^{2+}. When Mn^{2+} combines with E to form EMn^{2+}, the value of p must necessarily decrease and the enhanced effect of EMn^{2+} on the proton relaxation rate of water protons (Table I) must therefore result from a decrease in either τ_M or T_{1M}, or both. In general (Mildvan and Cohn, 1970) the enhancement in the case of macromolecules is principally due to a large decrease in the time constant for the relative rotation of Mn^{2+} and protons in the coordination sphere. This results in a significant decrease in T_{1M}.

The decreases in the enhancement values which occur when βMA and MES interact with EMn^{2+} (Table I) show that changes occur in the environment of the bound Mn^{2+}. Particularly striking is the greater than threefold reduction in enhancement obtained when MES combines with K^+E-Mn^{2+}. This has been termed type II enhancement behavior and it is typical of Mn^{2+}-activated enzymes for which direct evidence has been obtained for a metal bridge complex having either a simple (E–Mn^+–S) or cyclic structure (Mildvan,



1970). The rationalization of a cyclic metal bridge complex in terms of the probable mechanism of the β-methylaspartase reaction was discussed previously (Bright, 1967). However, changes in parameters other than p (see eq 8) can affect $1/T_{1p}$ when ligands interact with EMn^{2+}, and the enhancement data by themselves do not prove a bridging role for Mn^{2+} (Mildvan and Cohn, 1970). We are presently investigating the relaxation rates of the methyl protons of MES in the presence of EMn^{2+} (see, for example, Mildvan and Scrutton, 1967) in order to unequivocally test the cyclic metal bridge hypothesis.

References

Barker, H. A., and Smyth, R. D. (1960), *Biochem. Prepn. 8*, 586.

Barker, H. A., Smyth, R. D., Wilson, R. M., and Weissbach, H. (1959), *J. Biol. Chem. 234*, 320.

Bright, H. J. (1964), *J. Biol. Chem. 239*, 2307.

Bright, H. J. (1965), *J. Biol. Chem. 240*, 1198.

Bright, H. J. (1967), *Biochemistry 6*, 1191.

Bright, H. J., Lundin, R. E., and Ingraham, L. L. (1964), *Biochim. Biophys. Acta 81*, 576.

Cohn, M. (1963), *Biochemistry 2*, 623.

Cohn, M., and Townsend, J. (1954), *Nature (London) 173*, 1090.

Eisinger, J., Shulman, R. G., and Szymanski, B. M. (1962), *J. Chem. Phys. 36*, 1721.

Hsiang, M. W. (1967), Ph.D. Dissertation, University of Pennsylvania.

Hsiang, M. W., and Bright, H. J. (1967a), *J. Biol. Chem. 242*, 3079.

Hsiang, M. W., and Bright, H. J. (1967b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 26*, 605.
Hsiang, M. W., and Bright, H. J. (1969), *Methods Enzymol. 13*, 347.
Hughes, T. R., and Klotz, I. M. (1956), *Methods Biochem. Anal. 3*, 265.
Luz, Z., and Meiboom, S. (1964), *J. Chem. Phys. 40*, 2686.
Mildvan, A. S. (1970), Enzymes, New York, N. Y., Academic, 3rd ed (in press).
Mildvan, A. S., and Cohn, M. (1963), *Biochemistry 2*, 910.
Mildvan, A. S., and Cohn, M. (1970), *Enzymology 32* (in press).
Mildvan, A. S., and Scrutton, M. C. (1967), *Biochemistry 6*, 2978.
Williams, V. R., and Libano, W. Y. (1966), *Biochim. Biophys. Acta 118*, 144.

Kinetic Studies of the Nitrogenase-Catalyzed Hydrogen Evolution and Nitrogen Reduction Reactions*

Richard Silverstein† and W. A. Bulen‡

ABSTRACT: The nitrogenase enzyme from *Azotobacter vinelandii* can be considered as a complex of an Fe–Mo protein and an Fe protein that catalyzes H_2 evolution, the reduction of N_2 to NH_3, and the reduction of other substrates. All reactions are ATP dependent, releasing inorganic phosphate. Initial rates of H_2 evolution are reported as a function of both ATP and nitrogenase concentration. ATP rate profiles are sigmoidal. Nitrogenase profiles are concave upward becoming linear at higher concentrations. Computer simulation was used to correlate the rate and product data with a proposed reaction pathway involving: (1) a dynamic equilibrium between an associated enzyme species and its components, (2) the subsequent binding of two ATP molecules to the associated enzyme complex, and (3) breakdown of the resulting intermediate along two routes to yield products. ATP initial rate profiles for H_2 evolution in reactions conducted under N_2 were found to be hyperbolic, with the relative amounts of NH_3 to H_2 formation increasing at high ATP levels. The total number of electrons transferred to form products remained the same under N_2 as under argon at each ATP concentration studied. Introduction of the equilibrium constants for component association and the rate constants obtained in the treatment of H_2 evolution data provided, by computer calculation, an approximation of the concentration of intermediate reactive states of the enzyme leading to H_2 evolution and ammonia formation, respectively, as a function of ATP concentration.

Studies with the nitrogenase enzyme from *Azotobacter vinelandii* (Bulen and LeComte, 1966), *Clostridium pasteurianium* (Mortenson *et al.*, 1967), and more recently a number of other sources have demonstrated that the enzymatic activity results from the cooperative action of two protein components, an Fe–Mo protein and an Fe protein. Since neither protein has yet been shown to possess any catalytic activity alone, the components could be viewed as dissimilar subunits where the term applies to the dissociated species which may or may not be individually composed of monomeric units.

For activity the nitrogenase complex requires a source of electrons, which can be supplied *via* $Na_2S_2O_4$ (Bulen *et al.*, 1965) and ATP, which is hydrolyzed to ADP and P_i (Mortenson, 1964; D'Eustachio and Hardy, 1964; Bulen *et al.*, 1964).

A considerable amount of additional information has been accumulated relating to the reactions catalyzed by nitrogenase. In addition to N_2, it will also catalyze the reduction of C_2H_2, N_2O, N_3^-, HCN, CH_3NC, and certain analogs of some of these substrates. ATP hydrolysis studies (at 5mM ATP) have shown that (1) the rate of hydrolysis is constant with a given enzyme preparation and (2) the ratio of ATP hydrolyzed to electrons transferred to form product is independent of the substrate being reduced but exhibits a temperature dependence with ATP:$2e^-$ values ranging from 4.3 at 20° to 5.8 at 40° with values approaching 5 at 30° (Bulen and LeComte, 1966; Hadfield and Bulen, 1969). Kinetic data associated with these reactivities are, however, quite meager and it is the purpose of this report to contribute additional kinetic information obtained with the nitrogenase complex from *A. vinelandii*. Hardy *et al.* (1968) observed that C_2H_2 reduction was related to the enzyme concentration of a heated extract of N_2-grown *A. vinelandii* in a sigmoidal fashion and Moustafa and Mortenson (1967) reported that the initial rate of acetylene reduction by partially purified nitrogenase components from *C. pasteurianium* components was affected by ATP concentration.

In the absence of N_2 (or other reducible substrate) nitro-

* Contribution No. 386 of the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received May 6, 1970.* A preliminary report of this research was presented before the 158th National Meeting of the American Chemical Society, Sept 1969, New York, N. Y.

† Present address: Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kansas 66103.
‡ To whom to address correspondence.