thank Miss Noriko Ogasawara, The Institute for Applied Microbiology, University of Tokyo, for growing *Chlorella ellipsoidea* cells used in this study. They are also grateful to Dr. M. Funatsu for supplying us a sample of DFP–carboxypeptidase A and Drs. T. Murachi and N. Takahashi, Nagoya City University, Medical School, for their helpful discussions in connection with this study.

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Stabilization of Allosteric Adenosine Monophosphate Nucleosidase by Inorganic Salts, Substrate, and Essential Activator*

Vern L. Schramm† and L. I. Hochstein

ABSTRACT: Adenosine monophosphate nucleosidase (AMP nucleosidase, EC 3.2.2.4) has been purified over 80-fold from *Azotobacter vinelandii* OP and used to determine the mechanism of inactivation which occurs when the enzyme is placed in a low ionic strength environment. The kinetics of inactivation, together with results of gel filtration studies, indicate that the native enzyme exists in a polymeric form with an approximate molecular weight of 360,000. The enzyme is maintained in this form by a variety of inorganic salts as well as by the substrate (AMP) or essential activator ($MgATP^{2-}$). Removal of these protective agents results in a rapid loss of activity accompanied by the appearance of an inactive form of the enzyme having an apparent molecular weight of about 180,000. The inactive enzyme may be converted to the native form by the addition of salts, substrate, or activator. It is proposed that inorganic salts stabilize the native enzyme by neutralizing excess electrostatic charges in the molecule, which, when unshielded, disrupt the quaternary structure of the protein. Substrate and essential activator protect not by ionic shielding, but by combination at specific binding sites on the enzyme. Such combinations cause a conformational or charge distribution change which is sufficient to stabilize the quaternary structure in the absence of protection by inorganic salts. Comparison of initial velocity and stability experiments to both AMP and $MgATP^{2-}$ indicate that the substrate combines well with the enzyme either in the presence or absence of essential activator. Thus the function of $MgATP^{2-}$ must be to provide a catalytically active conformation rather than to form a binding site for the substrate.

In recent years a wide variety of allosteric enzymes have been studied kinetically with the results usually being interpreted in terms of the hypotheses proposed by either Monod *et al.* (1965) or Koshland *et al.* (1966). Because these hypotheses have been based only on the binding of ligands, with no consideration given to kinetic effects (rate changes as a consequence of multiple ligand bindings), such interpretations are, at best, only approximations to the actual mechanism of allo-

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steric enzyme catalysis (Frieden, 1967). Interpretation of allosteric kinetics[1] according to individual reaction mechanisms was proposed by Atkinson *et al.* (1965) and has had successful application in several cases (Sanwal and Cook, 1966; Schramm and Morrison, 1969), although results of kinetic studies cannot be used for information regarding enzyme structure or conformation. The most informative investigations of allosteric enzymes have been those in which kinetic, thermodynamic, and structural studies with homogeneous enzyme preparations are correlated to determine the mechanism of action (*e.g.*, Changeux *et al.*, 1968; Gerhart and Schachman, 1968).

With such considerations in mind, an investigation of the allosteric AMP nucleosidase from *Azotobacter vinelandii* OP has been initiated to determine its mechanism of action. AMP nucleosidase catalyzes the hydrolysis of AMP in the presence of divalent cations and ATP to give ribose 5-phosphate and adenine. Both the divalent metal ion and ATP are essential for catalysis, although ATP is unchanged during the course of the reaction (Hurwitz *et al.*, 1957). Plots of initial velocity as a function of ATP have been shown to be sigmoidal, while those obtained as a function of substrate, AMP, were hyperbolic (Yoshino *et al.*, 1967; Yoshino, 1970). A further property of the enzyme is its ability to undergo reversible dissociation in the presence of either ATP or AMP and upon changes of salt concentration (Yoshino *et al.*, 1968; Ogasawara *et al.*, 1970). These properties, plus the observation that the enzyme is strongly inhibited by low concentrations of inorganic phosphate, suggest that AMP nucleosidase may play a regulatory role in *Azotobacter vinelandii* OP.

When attempts were made to purify AMP nucleosidase by conventional techniques such as ion-exchange or gel filtration chromatography, the enzyme rapidly lost activity. This could be prevented by including relatively high concentrations of inorganic salts (0.25 M K_2SO_4 was most commonly used) in the buffers present during purification. This, however, imposed limitations on the purification techniques which could be used and only an 80-fold purification could be achieved. Enzyme prepared in this manner has been used to investigate the mechanism of enzyme inactivation, and has revealed the basis of the salt-induced stability as well as other methods by which the enzyme may be stabilized. The results of this study may be used to facilitate further purification needed for the preparation of a homogeneous enzyme.

Materials and Methods

Materials. Adenine and the sodium salts of AMP and ATP were products of P-L Biochemicals. Ribose 5-phosphate, dithiothreitol, and phenylmethylsulfonyl fluoride were obtained from Calbiochem. Tris, calcium phosphate gel, EDTA, crystalline yeast alcohol dehydrogenase, and xanthine oxidase were purchased from Sigma. Triethanolamine and 2,9-dimethyl-1,10-phenanthroline were Baker Analyzed reagents. DEAE-81-cellulose paper was supplied by Whatman. Crystalline catalase and creatine kinase were purchased from Boehringer; urease and lactic dehydrogenase were products of Worthington.

Nucleotides were dissolved in distilled water and adjusted to pH 7.6 with 1 N NaOH, and their concentrations determined according to the method of Bock *et al.* (1956). Triethanolamine was purified by redistillation *in vacuo* before use. All other chemicals were reagent grade commercial products and were used without further purification.

Measurement of Enzyme Activity. AMP nucleoside activity was determined by the formation of reducing sugar (ribose 5-phosphate) from AMP in the presence of the essential activators $MgCl_2$ and ATP. Reducing sugar was determined by a modification of the method of Dygert *et al.* (1965). The enzymatic activity was determined at 30° in 0.10 or 0.25 ml of reaction mixture which contained: triethanolamine-HCl (0.1 M, pH 8.0), AMP (4 mM), ATP (7 mM), and $MgCl_2$ (7 mM). Equimolar concentrations of $MgCl_2$ and ATP were assumed to form the $MgATP^{2-}$ complex, using the reported stability constant of 73,000 M^{-1} in 0.1 M triethanolamine (pH 8.0; O'Sullivan and Perrin, 1964). The reaction was initiated by the addition of from 2 to 50 μl of enzyme. The reaction was terminated by the addition of 0.30 ml of the reducing sugar reagent (which contained, in 400 ml: Na_2CO_3 (16 g), glycine (6.4 g), and $CuSO_4 \cdot 5H_2O$ (0.18 g). After addition of 0.30 ml of the second reducing sugar reagent (which contained, in 400 ml: 2,9-dimethyl-1,10-phenanthroline (0.48 g), adjusted to pH 3.0 with HCl), the tubes were placed in a boiling-water bath for 8 min. After rapidly cooling the tubes to 30°, the yellow color remained stable for at least 1 hr. The millimolar extinction coefficient was found to be 27.0 at 450 mμ using ribose 5-phosphate as the standard. Under the above conditions, initial velocity was maintained during 20% of substrate hydrolysis. However, in practice, no more than 4% of the substrate was hydrolyzed, thus assuring that rates would not deviate from initial velocities due to substrate exhaustion or end-product inhibition

A unit of activity was taken to be the amount of enzyme which released 1 μmole of ribose 5-phosphate per min under the above assay conditions.

Fractionation with Ammonium Sulfate. The weight (w) of ammonium sulfate required to give the desired saturation was calculated from the formula $w = 0.515V(S_2 - S_1)/(1.0 - 0.292S_2)$, where V is volume in milliliters, w is in grams, and S_1 and S_2 are the initial and desired degrees (0–1.0) of saturation at 0° (Kunitz, 1952; Noltmann *et al.*, 1961).

Determination of Protein. Protein was estimated either with the method of Lowry *et al.* (1951) or by the microbiuret method (Goa, 1953) using bovine serum albumin as the standard.

Growth of Azotobacter vinelandii OP. Cells (obtained from Dr. D. C. Yoch, Department of Cell Physiology, University of California, Berkeley) were grown in modified Burk's medium (Newton *et al.*, 1953) which contained the following additions per liter of distilled water: sucrose (20 g), NaCl (0.1 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), $Fe_2(SO_4)_3 \cdot 3H_2O$ (10 mg), $FeCl_2 \cdot 4H_2O$ (3.5 mg), and $Na_2MoO_4 \cdot 2H_2O$ (0.25 mg). Starter cultures were grown at 30° in 500 ml of medium in 2-l. flasks. Three liters of starter culture in the late-log phase were used to inoculate 200 l. of freshly prepared, nonsterile medium in a 220-l. Nalge container. Aeration and stirring were achieved with a centrifugal-type pump (Eastern Industries, Model SD-11) immersed in the medium. Filtered air (12 l./min) and medium were drawn into the pump and discharged as a stream of medium and finely dispersed bubbles. Temperature was maintained at 30° and cells were harvested in a Sharples refrigerated centrifuge after 24 hr, at which time the culture had attained a turbidity of 200 Klett units using a no. 42 blue filter. The cells (about 400 g) were frozen and stored at −10° at which temperature

[1] The term "allosteric enzyme" as used in this paper refers to enzymes which show (a) marked activation or inhibition by naturally occurring compounds other than substrates or products, and (b) a sigmoidal plot of initial velocity as a function of either substrate, activator, or inhibitor concentration. Thus the term "allosteric kinetics" refers to kinetics exhibited by enzymes with the above properties.

AMP nucleosidase activity remained constant for more than 4 months.

Results

Preparation of AMP Nucleosidase; Initial Extract. Unless otherwise stated, all purification steps were carried out at from 0 to 5° in buffers containing EDTA (10^{-4} M), dithiothreitol (10^{-4} M), and phenylmethysulfonyl fluoride (3×10^{-6} M). Cells (32 g) were thawed and diluted with 0.05 M Tris-HCl (pH 8.0) buffer containing 0.25 M K_2SO_4 (Tris-K_2SO_4 buffer) to give a total volume of 75 ml. The suspension was passed through an Aminco French pressure cell at 15,000–25,000 psi, diluted with additional Tris-K_2SO_4 buffer to 150 ml, and centrifuged at 30,000*g* for 10 min. Dark brown supernatant (145 ml) was recovered and designated as the initial extract.

Heat Treatment. The initial extract was diluted to 250 ml with the Tris-K_2SO_4 buffer and heated to 60° over a period of 5 min in a stainless steel centrifuge tube. After an additional 10 min at that temperature, the tube was cooled in ice and the solution centrifuged at 30,000*g* for 10 min. The AMP nucleosidase activity was recovered in 194 ml of turbid brown supernatant.

Protamine Sulfate Treatment. The pH of the supernatant was adjusted to 6.8 by the slow addition of 1 N HCl, accompanied by rapid stirring. Protamine sulfate (9 ml, 2.5%, pH 6.0) was added dropwise to the stirred solution, followed by centrifugation at 11,000*g* for 10 min to give 212 ml of supernatant.

Ammonium Sulfate Fractionation. The supernatant from above was adjusted to pH 7.5 with the cautious addition of 1 N NaOH, and the stirred solution brought to 0.42 saturation with solid $(NH_4)_2SO_4$. Following centrifugation (30,000*g* for 10 min) the supernatant was brought to 0.47 saturation and centrifuged as above. The resulting pellet was dissolved in Tris-K_2SO_4 buffer to give 24 ml.

Sodium Citrate Fractionation. Sodium citrate·$2H_2O$ (12 g) was dissolved in the 24 ml of ammonium sulfate fraction, and after stirring at 0° for 30 min, was diluted by the addition of 10 ml of distilled H_2O, followed by an additional 10-min stirring. The precipitate was collected by centrifugation at 75,000*g* for 15 min. The pellet was extracted with 10 ml of 0.65 saturated sodium citrate, followed by 0.55 saturated sodium citrate, both in 0.05 M Tris (pH 8.0) containing 0.25 M K_2SO_4. Centrifugations were done as above; the pellet remaining after both extractions was dissolved in Tris-K_2SO_4 buffer to give 10 ml and was used for the next step.

Calcium Phosphate Gel Fractionation. The sodium citrate fraction was diluted to 16 ml with Tris-HCl (0.05 M, pH 8.0) buffer containing 2 M NaCl, and the resulting solution dialyzed overnight against 1.0 l. of the same buffer. Calcium phosphate gel (23% solids, 0.8 g) was added and the mixture stirred for 5 min. The gel was sedimented by centrifugation at 3000*g* for 5 min and the supernatant discarded. The gel was washed for 10 min with 10-ml portions of the Tris–NaCl buffer (pH 8.0) described above, containing 10, 20, 40, and 80 mM potassium phosphate (pH 7.0), and centrifuged at 3000*g* for 5 min. The enzyme appeared in the 10 and 20 mM phosphate washes; they were combined and concentrated to 5 ml in an Amicon Diaflow concentrator.

Gel Filtration. The concentrated enzyme was applied to a column of Sephadex G-200 (2.5 × 100 cm) previously equilibrated with Tris-K_2SO_4 buffer. The column was washed with the same buffer and the enzyme fractions of the highest specific activities were collected and concentrated to give a final volume of 8.5 ml. A summary of the purification procedure is shown in Table I.

TABLE I: The Purification of AMP Nucleosidase from *A. vinelandii* OP.[a]

Fraction	Vol (ml)	Total Protein (mg)	Total Units (μmoles/min)	Sp Act. (μmoles/min per mg)
Initial extract	145	5220	122	0.023
Heat treatment	194	1690	91	0.054
Protamine sulfate treatment	212	1420	90	0.063
$(NH_4)_2SO_4$ fractionation	24	252	54	0.21
Sodium citrate fractionation	10	80	33	0.41
Calcium phosphate gel fractionation	20	19	19	1.0
Sephadex G-200 chromatography	8.6	5.3	10	2.0

[a] Wet weight of *Azotobacter* was 32 g. Details are given in the text.

Properties of the Purified Enzyme. The Sephadex G-200 fraction was free of myokinase, ATPase, and adenase activities. Prolonged incubation of the enzyme in the reaction mixture showed no changes in the ATP concentration (as determined by coupling the hexokinase and glucose 6-phosphate dehydrogenase reactions). In the absence of $MgCl_2$ and ATP, no reducing sugar was released from AMP. The products of enzymatic action on AMP ran in identical positions with adenine and ribose 5-phosphate when chromatographed on DEAE-cellulose paper in 0.2 M ammonium formate (pH 3.2) according to the method of Morrison (1968). The enzyme lost 50% of the original activity after 10-months storage at 3° in Tris-K_2SO_4. Freezing the enzyme had no effect on activity and it remained stable for 2 months at −10° in Tris-K_2SO_4 buffer.

Protection by Inorganic Salts. Dilution of the purified enzyme into varying concentrations of inorganic salts resulted in a rapid loss of activity at low salt concentrations, with increasing protection at higher salt concentrations (Figures 1a–e). This protection was complete at concentrations of K_2SO_4 or KCl greater than 200 mM, while a similar concentration of K_2HPO_4 (pH 8.0) resulted in an increase of activity. In contrast to the above results, increasing concentrations of either $MgCl_2$ or $MgSO_4$ gave good protection at low concentrations (10 mM) but at concentrations where other salts gave complete stability, incomplete protection was obtained. Although experimental conditions prevented inactivation studies in the total absence of salt, plots of per cent activity remaining at 30 min as a function of K_2SO_4 concentration (Figure 1f) passed through the origin, suggesting that complete loss of activity could be expected in the absence of protective ions. Further, this result suggests that the presence of 0.05 M Tris-HCl (pH 8.0), the dilution buffer for the inactivation experiments, offers little protection to the enzyme. Additional studies at a fixed concentration (0.5 M) of a variety of salts including $NaC_2H_3O_2$, $NaNO_3$, NaCl, NaBr, LiCl, RbCl, and CsCl showed that each was capable of stabilizing the enzyme.

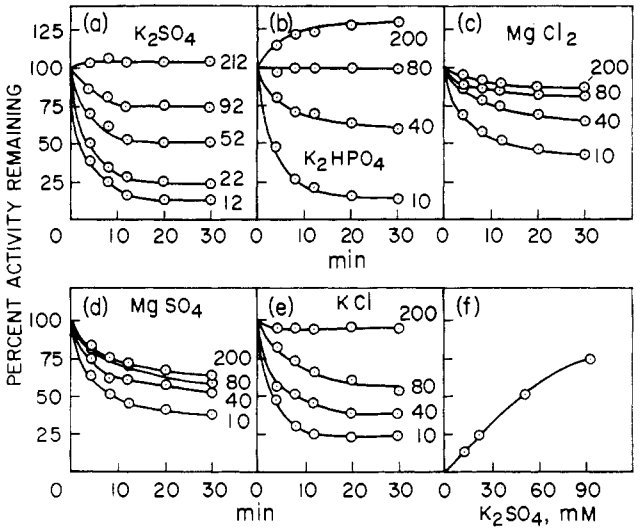


FIGURE 1: Effect of diluting the enzyme into varying concentrations of protective salts. Inactivation experiments were carried out at 30° in conical centrifuge tubes containing the desired salt solution in 0.05 M Tris buffer (no additives), pH 8.0. Enzyme (0.02 ml, stored in 0.25 M K_2SO_4–0.05 M Tris buffer) was added to 0.38 ml of the salt solution, and loss of activity monitored by withdrawing 0.04-ml samples at appropriate time intervals and incubating for 20 min in 0.25 ml of the reaction mixture described in the text. Concentrations of all salts are given as millimolar. Part a shows the effect of varying concentrations of K_2SO_4 on stability. The 12 mM curve represents the amount of K_2SO_4 carried over from the stock enzyme solution to the dilution buffer. Parts a–e show the protective effects of K_2HPO_4 (pH 8.0), $MgCl_2$, $MgSO_4$, and KCl, the concentrations shown not including the 12 mM K_2SO_4 present as a result of the enzyme storage buffer. Part f illustrates the per cent of activity remaining after 30 min at 30° as a function of total K_2SO_4 concentration. Per cent activity remaining is expressed relative to a sample taken 15 sec after dilution.

The above results suggest an equilibrium existing between active and inactive forms of the enzyme, with the equilibrium position depending on the concentration of salt used for protection. In the absence of salts, the enzyme is completely inactive, while intermediate levels give a distribution between the two forms. K_2HPO_4 (pH 8.0) causes the most complete conversion into the active form, while magnesium salts are incapable of shifting the equilibrium completely to the active enzyme form.

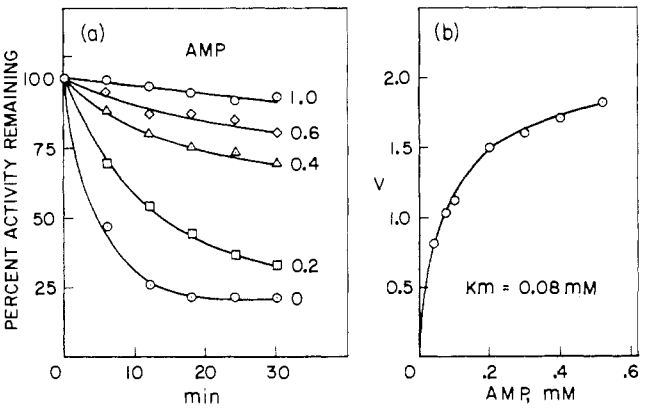


FIGURE 2: Stability and initial velocity as a function of substrate (AMP) concentration. Stability studies shown in (a) were carried out as described in the legend to Figure 1, with AMP concentration in millimolar and per cent remaining activity reported relative to 15 sec after dilution. The results in part b illustrate the initial velocity of AMP nucleosidase as a function of AMP concentration at a fixed concentration of the essential activator, $MgATP^{2-}$ (2.0 mM). Initial velocity (v) is expressed as μmoles/min per mg of protein. The Michaelis constant (K_m) was obtained from a double-reciprocal plot of the data.

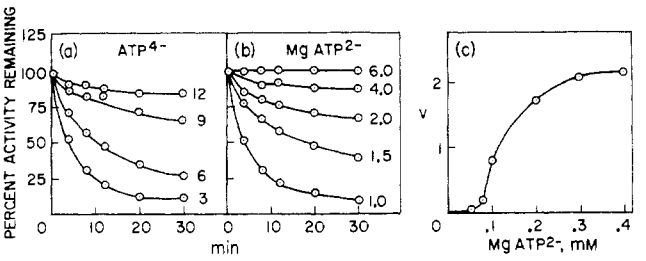


FIGURE 3: Stability and initial velocity as functions of free ATP^{4-} (a) and $MgATP^{2-}$ (b) were carried out as described in the legend to Figure 1, with concentrations in millimolar and per cent remaining activity reported relative to 15 sec after dilution. The results in part c illustrate the initial velocity of AMP nucleosidase as a function of $MgATP^{2-}$ concentration at a fixed concentration of substrate (2.0 mM). Initial velocity (v) is expressed as μmoles/min per mg of protein.

Protection by Substrate (AMP). Dilution of enzyme into various concentrations of substrate, AMP, in the absence of activator had the effect of stabilizing the enzyme as shown in

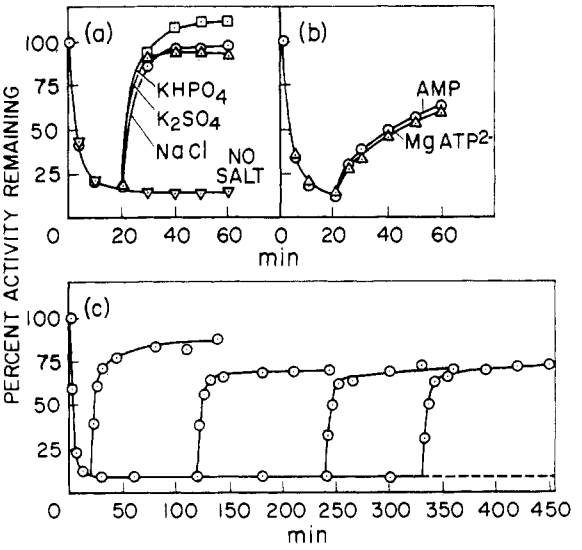


FIGURE 4: Inactivation and reactivation of AMP nucleosidase. Enzyme was inactivated by dilution into Tris (0.05 M, pH 8.0) buffer as described in the legend to Figure 1. Reactivation of activity in part a was accomplished by the addition at 21 min of: 4 M NaCl in Tris 0.05 M (pH 8.0) to give a final concentration of 0.5 M, ○; 0.7 M K_2SO_4 in the Tris buffer to give a final concentration of 0.25 M, △; or 1.0 M K_2HPO_4 (pH 8.0) in the Tris buffer to give a final concentration of 0.15 M, □. Reactivation in part b was accomplished by the addition at 2 min of: 50 mM AMP to give a final concentration of 4 mM, ○; or 25 mM $MgATP^{2-}$ to give a final concentration of 7 mM, △. Reactivation in part c was accomplished by adding a sample of diluted (inactivated) enzyme at the appropriate time to a conical tube containing sufficient 4 M NaCl in the Tris buffer to give a final concentration of 0.5 M. Activity was assayed in all cases as described in the legend to Figure 1, with corrections being made for dilution and inhibition of activity by added salt solutions. The added salts gave the following inhibitions in the assay mixture: NaCl, 9%; K_2SO_4, 12%; K_2HPO_4, 32%. Per cent of activity remaining is expressed relative to that at 15 sec after dilution.

Figure 2a. The protection curves are similar to those obtained with inorganic salts; however, with substrate, nearly complete protection is obtained at a concentration of only 1 mM. At this concentration, which is 12 times the K_m for AMP in the presence of saturating activator concentration (Figure 2b), the substrate site(s) will approach saturation if the enzyme–substrate combination is not dependent on the presence of activator. Thus it appears that AMP is protecting by combination at the active site(s), and that the presence of $MgATP^{2-}$ is not essential for the formation of the enzyme–substrate complex.

Relative Protection by ATP^{4-} and $MgATP^{2-}$. As both Mg^{2+} and ATP^{4-} must be added for enzymatic activity, it was of interest to determine if either or both of the ionic species, ATP^{4-} and $MgATP^{2-}$, could protect the enzymatic activity in the absence of high salt concentrations. The results of dilution into varying concentrations of free ATP^{4-} are shown in Figure 3a, while a similar experiment with $MgATP^{2-}$ is shown in Figure 3b. The data indicate that both species are capable of protecting the enzyme, but $MgATP^{2-}$ is effective at much lower concentrations (about one-third) than is ATP^{4-}, a finding which is not surprising in view of the fact that in most enzymes requiring both Mg and ATP as reactants, the active species is the $MgATP^{2-}$ complex (Schramm and Morrison, 1968; London and Steck, 1969; Kemp, 1969).

In contrast to the protection of AMP nucleosidase by substrate levels of AMP (Figure 2), protection by the essential activator, $MgATP^{2-}$, requires concentrations above 4 mM to give complete protection. Conversely, the results of Figure 2c indicate that the concentration of $MgATP^{2-}$ required for half-maximum velocity in the presence of high (2 mM) concentrations of substrate is only 0.12 mM. Even at 12 times that concentration, virtually no protection is present for the enzyme. This would indicate that while $MgATP^{2-}$ is capable of combining with the enzyme, it combines much better in the presence of AMP than in its absence.

Reversibility of Inactivation. The foregoing experiments had indicated that a salt- (or AMP, or $MgATP^{2-}$) dependent equilibrium exists between active and inactive forms of AMP nucleosidase. If this proposition is correct, it should be possible to reverse the equilibrium by increasing the concentration of salts or by adding substrate or activator to the inactive form of the enzyme. The results of such experiments are shown in Figure 4, in which it may be seen that K_2SO_4, K_2HPO_4, and NaCl (Figure 4a) were effective in reactivating the enzyme which had been inactivated by dilution into salt-free buffer. In addition, both AMP or $MgATP^{2-}$ were capable of reactivating the enzyme (Figure 4b), although the rate of reactivation is much slower than that caused by salt. The inactivation–reactivation reaction was further examined by determining if the inactive form of the enzyme was stable, or was a transient form which leads to an irreversibly inactive (denatured) enzyme. Figure 4c illustrates that even after 5.5 hr at 30° in the inactive form, the enzyme is capable of being reactivated to 70% of its original activity.

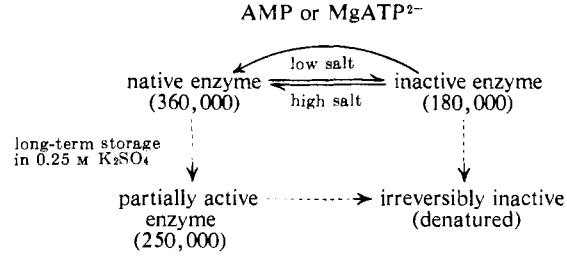
Molecular Weight Estimation of Native and Inactivated Enzyme. Samples of AMP nucleosidase were mixed with catalytic quantities of xanthine oxidase, catalase, yeast alcohol dehydrogenase, and creatine kinase as needed for molecular weight markers and passed through a (1.2 × 11.4 cm) column of Sephadex G-200. Two molecular weight estimations as described by Andrews (1964) in 0.05 M Tris gave values of 372,000 and 355,000 while a similar experiment in Tris (0.05 M, pH 8.0) with 1 mM AMP gave an apparent molecular weight of 360,000. One of these determinations is shown in Figure 5a, while Figure 5b demonstrates a similar experiment in which the column had first been equilibrated against the Tris buffer (0.05 M, pH 8.0) containing only 0.025 M K_2SO_4. In this experiment, fractions were collected in tubes containing sufficient 4 M NaCl to give a final concentration of 0.5 M NaCl, which is sufficient to reactivate the enzyme (see Figure 4). Two such experiments gave molecular weight values of 155,000 and 186,000 which is consistent with a dissociation of the enzyme molecule caused by dilution into low salt. The reversibility of this dissociation was demonstrated by applying an enzyme sample which had been inactivated by dilution into low salt, reactivated by the addition of K_2SO_4 to give 0.25 M, and passed through the column equilibrated with Tris buffer (0.05 M, pH 8.0) containing 0.25 M K_2SO_4. The resulting activity was eluted in a symmetrical peak with an apparent molecular weight of 390,000.

In contrast to the symmetrical elution patterns seen under the conditions mentioned above, gel filtration in 0.25 M K_2SO_4 of an enzyme preparation that had been aged for 11 months at 3° in 0.25 M K_2SO_4 resulted in an asymmetrical elution pattern with a main peak and shoulder corresponding to apparent molecular weights of 380,000 and 250,000, respectively. Thus it appears that the enzyme is capable of existing in at least three different molecular weight forms: active 360,000 and 250,000 forms as well as the reversibly inactive 160,000 molecular weight enzyme.

Discussion

Results of inactivation and stability studies with partially purified AMP nucleosidase have indicated that the enzyme may exist in several polymeric forms and undergo a reversible loss of activity. The experimental results are in agreement with the mechanism of inactivation summarized in Scheme I,

SCHEME I



where the native enzyme is maintained in the active form by relatively high concentrations of inorganic salts. Protection of protein structure by inorganic salts has been frequently observed (Von Hippel and Wong, 1964), and such effects usually attributed to ionic shielding, whereby the acidic amino acid side chains are neutralized by the presence of inorganic cations. Such neutralization is nonspecific with respect to inorganic salts used, and such is the case with AMP nucleosidase. Further evidence to support this mechanism comes from the observation in both this and other systems (Denney and Monk, 1951) that Mg^{2+} is more effective in ionic shielding at low concentrations than are similar concentrations of monovalent ions. However, with AMP nucleosidase, higher Mg^{2+} concentrations prevent complete stabilization of the enzyme, and at the present time, the mechanism of this magnesium-specific effect remains unknown.

Reactivation and gel filtration experiments have confirmed the reversibility and molecular weight change involved in the

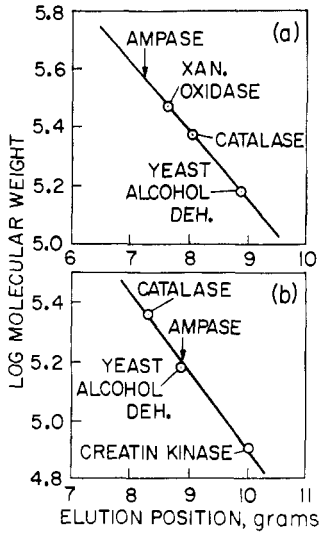


FIGURE 5: Elution positions of AMP nucleosidase in 0.05 M Tris (pH 8.0) containing either 0.25 M K_2SO_4 (a) or 0.025 M K_2SO_4 (b). AMP nucleosidase (15 μg) was mixed with xanthine oxidase (500 μg), catalase (100 μg), yeast alcohol dehydrogenase (50 μg), or creatine kinase (25 μg) as required and diluted to 0.2-ml total volume with the appropriate elution buffer. Fractions of about 0.3 g were collected and peaks were located by their enzymatic activity. The peaks of the elution patterns were used as the position of elution for plots of elution position *vs.* log molecular weight.

active to inactive enzyme transitions when salt concentrations are varied. This reversible dissociation process is thought to be due to the presence of electrostatic repulsion when excess negative charges are uncovered in the molecule. Neutralization of these charges allows the dissociation to be reversed. Further evidence to support this mechanism is provided by the similarity in rates of inactivation in the absence and reactivation in the presence of salts.

In contrast to the results obtained with inorganic salts, reactivation of the inactive enzyme by substrate or essential activator clearly occurs by a different mechanism as evidenced by the much slower rate of reactivation in the presence of these compounds, as well as the fact that ionic shielding could not be achieved by the concentrations of these compounds used. The simplest interpretation of protection by AMP and $MgATP^{2-}$ is that they combine at the substrate and allosteric sites, respectively. The experimental evidence does not, however, distinguish between the above explanation and the possibility that at the high concentrations of activator needed for protection, $MgATP^{2-}$ may be combining at the substrate site, which must be somewhat similar as judged by the similarity between the $MgATP^{2-}$ and AMP^{2-} molecules. In any case, these combinations must cause a conformational change which decreases electrostatic forces in the enzyme sufficiently to prevent dissociation from occurring. Further, it should be noted that in the presence of the high concentrations of both AMP and $MgATP^{2-}$ used in the assay mixtures, there is neither reactivation of inactive enzyme nor loss of activity from the active form, which suggests that when both allosteric and substrate sites are filled, the enzyme can neither reassemble nor dissociate.

In addition to the reversible dissociation reaction caused by the lack of protective compounds, two other forms of enzyme can be demonstrated. One of these is the denatured enzyme which slowly arises from the reversibly inactive enzyme (Figure 4c) or from enzyme stored in 0.25 M K_2SO_4 buffer solutions (11 months at 3°). The other form appears during this long-term storage also, but retains partial activity and exhibits an apparent molecular weight of 250,000 upon gel filtration. The presence of enzyme species with molecular weights of approximately 360,000, 250,000, and 180,000 indicate that the native enzyme consists of four or more subunits.

Several theories have been proposed whereby allosteric kinetic data could be caused by substrate or activator induced association–dissociation reactions (Nichol *et al.*, 1967; Frieden, 1967), and several allosteric enzymes do seem to fit into such a category (*e.g.*, Frieden and Colman, 1967; Long *et al.*, 1970). The allosteric effects (*i.e.*, sigmoidal plots of initial velocity against $MgATP^{2-}$ concentration) obtained with AMP nucleosidase are difficult to explain in terms of these theories, as sigmoidal initial rate curves are obtained under conditions (>1 mM AMP) where the enzyme would be expected to be in the fully active form. Careful initial velocity measurements under these conditions show no variation from linearity, which, if present, could indicate an association–dissociation reaction occurring during the course of the reaction. These observations do not preclude the possibility that dissociation effects may occur at low concentrations of both activator and substrate; however, they do indicate that dissociation is not a *necessary* condition for sigmoidal rate curves with $MgATP^{2-}$.

The results reported here differ somewhat from those published recently (Yoshino, 1970; Ogasawara *et al.*, 1970) in which the enzyme was purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis and found to have a molecular weight of 240,000 in the presence of protective salts and 120,000 in their absence. In the above-mentioned studies, both the associated and dissociated enzyme forms had equivalent activity. These discrepancies may be accounted for by differences in the method of purification. The procedure reported in this paper was carried out exclusively in the presence of protective salts, while in the previous report (Yoshino, 1970), the cells were sonicated in distilled water and the enzyme had been dialyzed several times in the absence of protective concentrations of salt. Such treatments may have led to a different reassembly of subunits. Recent work on a crystalline AMP nucleosidase prepared in the presence of AMP for protection is in agreement with the 360,000 molecular weight for native AMP nucleosidase (V. L. Schramm, in preparation).

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A Calorimetric Study of the Chymotrypsinogen Family of Proteins*

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ABSTRACT: Several types of calorimetric measurements have been performed on three members of the chymotrypsinogen family of proteins. The heat of protonation of the carboxyl groups of α-chymotrypsin in its best folded state indicates no apparent abnormality in the titration behavior. Such is not the case for chymotrypsinogen A for which an unusually large heat change (~30 kcal/mole) is observed at low pH. This effect is not related to thermal unfolding but is highly indicative of one or more buried carboxyl groups in this protein. The enthalpy changes for the reversible thermal unfolding of chymotrypsinogen at 50° and α-chymotrypsin and dimethionine sulfoxide chymotrypsin at 25 and 40° have been estimated. These estimated values are in good agreement with those obtained from van't Hoff analysis of equilibrium data. This agreement supports the validity of the two-state approximation for the transition. Apparent molar heat capacities for the above three protein species have been determined in their folded and unfolded states. A large increase in heat capacity upon unfolding is observed. Differences in heat capacity between the species in their best folded states are most apparent in the temperature dependence of the heat capacity which are probably related to conformational differences. In the unfolded state the heat capacity was found to be identical for all three species. This latter observation is strong evidence that the unfolded state is thermodynamically similar for all three species.

Present understanding of thermodynamic aspects of protein conformation and conformational changes has been derived principally from van't Hoff studies of reversible thermally induced unfolding at acid pH. Several conclusions which are consistent with the data can be drawn. In some cases such as lysozyme (O'Reilly and Karasz, 1970), ribonuclease (Tsong *et al.*, 1970), and chymotrypsinogen (Jackson and Brandts, 1970) calorimetric data are also available and are in basic agreement with these conclusions listed below. (1) Many globular proteins undergo reversible thermal unfolding *via* a two-state transition.[1] That is, the temperature-dependent equilibrium is an equilibrium between two and only two conformational states; under experimental conditions states of intermediate folding do not exist in any appreciable concentration (Lumry *et al.*, 1966). (2) Unfolding reactions of proteins are accompanied by enthalpy and entropy changes which are extremely temperature dependent because of a large heat capacity difference between the two forms of the protein. This

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[1] The use of the term two state is used to designate an equilibrium between two macroscopic distributions. These two states are not necessarily two well defined structures but can include structural variations within molecules of a given state. Furthermore, the existence of intermediate states is not completely ruled out, but only that the population of such intermediate states is negligible. Details of the meaning of this approximation are thoroughly discussed by Lumry *et al.* (1966) and Jackson and Brandts (1970).