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Purification, Crystallization, and Subunit Structure of Allosteric Adenosine 5′-Monophosphate Nucleosidase†

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ABSTRACT: Adenosine monophosphate nucleosidase (adenosine monophosphate phosphoribohydrolase, EC 3.2.2.4) has been purified from *Azotobacter vinelandii* strain OP. The purified enzyme preparation was homogeneous as judged by analytical ultracentrifugation and polyacrylamide gel electrophoresis. Following purification, the enzyme was crystallized from ammonium sulfate solutions. The molecular weight of the enzyme was approximately 370,000 as determined from gel filtration and sedimentation velocity experiments, while a molecular weight of approximately 325,000 was obtained from the Archibald approach to equilibrium method. Results of polyacrylamide gel electrophoresis in sodium dodecyl sulfate or 9 M urea, as well as analysis of amino acid composition, suggest that the enzyme consists of similar polypeptide chains having a molecular weight of approximately 57,000. The crystalline enzyme differs from previously described preparations of AMP nucleosidase in that the substrate- or salt-induced association–dissociation reaction does not occur.

Adenosine monophosphate nucleosidase catalyzes the hydrolysis of AMP[1] to yield ribose 5-phosphate and adenine:

$$\text{AMP} \xrightarrow{\text{MgATP}} \text{adenine} + \text{ribose 5-phosphate}$$

The enzyme was first described by Hurwitz *et al.* (1957) who showed that both magnesium and ATP were required for catalysis, and that ATP is not altered by the enzyme. Studies have indicated that the enzyme exhibits Michaelis–Menten kinetics with substrate, but that plots of initial velocity as a function of MgATP are strongly sigmoidal (Yoshino *et al.*, 1967; Schramm and Hochstein, 1971). Thus the enzyme may be considered as being allosteric.[2]

Recent reports of Yoshino (1970) and Ogasawara *et al.* (1970) have described the preparation of homogeneous AMP nucleosidase from *Azotobacter vinelandii*. However the enzyme obtained by these workers was unstable in the absence of high salt concentrations and could not be completely stabilized even in the presence of 0.4 M K_2SO_4. The molecular weights of this preparation were reported to be 240,000 in the presence of substrate, activator or high ionic strength and 120,000 in their absence. These results differ from those obtained by Schramm and Hochstein (1971) with an 80-fold-purified preparation of this enzyme, which showed apparent molecular weights of 360,000 and 180,000 under conditions similar to those described above. With this latter preparation a 240,000 molecular weight form appeared only after prolonged ageing of the 360,000 molecular weight enzyme (Schramm and Hochstein, 1971). In order to resolve these discrepencies, physical and chemical studies were performed on a

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[1] Molecules carrying charges such as $MgATP^{2-}$, ATP^{4-}, and AMP^{2-} have been written without their respective charges. Other abbreviations are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMSF, phenylmethylsulfonyl fluoride.

[2] "Allosteric" 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.

crystalline preparation of AMP nucleosidase, purified 1300-fold from initial extracts. The results of these studies are described.

Materials and Methods

Materials. Protein molecular weight standards were obtained from Boehringer, Sigma, and Worthington, and were the highest purity available. Nucleotides were obtained from P-L Biochemicals. Guanidine·HCl was obtained from Eastman and was decolorized by heating with charcoal according to the methods of Edelhoch (1967) before use. Triethanolamine (Eastman) was purified by distillation *in vauco* and stored at 2° in the dark. Glycine ethyl ester·HCl and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide·HCl were obtained from Pierce. Glycine ethyl ester-*1*-^{14}C·HCl as obtained from New England Nuclear. All other chemicals were of reagent grade and were used without further purification.

Measurement of Activity. AMP nucleosidase activity was determined at 30° by the formation of reducing sugar from AMP (4 mM) in the presence of a saturating concentration of MgATP (7 mM) in 0.1 M triethanolamine (pH 8.0) was previously described by Schramm and Hochstein (1971). Enzymatic activity is expressed as micromoles of reducing sugar formed per minute per milligram of protein.

During purification, protein concentrations were estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. The protein concentration of purified AMP nucleosidase was calculated from the tyrosine absorption in 6 M guanidine·HCl using the absorption constants of Edelhoch (1967). Total protein was then calculated using the relative frequency of tyrosine from amino acid analysis. The weight of ammonium sulfate required to give the various degrees of saturation was calculated according to the method of Noltman *et al.* (1961).

A. vinelandii OP cells were grown on the nitrogen free media described by Newton *et al.* (1953) in 200-l. batches as previously described (Schramm and Hochstein, 1971), except that the medium was sterilized before use by the direct addition of 0.2% β-propiolactone.

Molecular Weight Studies. Sedimentation velocities of the purified enzyme were determined in a Spinco Model E analytical ultracentrifuge at 20° using Tris·HCl buffer (0.1 M, pH 8.0) containing EDTA (10^{-4} M), dithiothreitol (10^{-4} M), and phenylmethylsulfonyl fluoride (3×10^{-6} M) both in the presence and absence of 2 mM AMP. The density (ρ) of the buffer at 20° was 0.998 g/cm^3 and its viscosity (η) was 1.002 at 20° as measured with a Cannon Ubbelohde viscometer.

The Stokes' radius of AMP nucleosidase was determined as described by Andrews (1970) using ferritin, urease, apoferritin, xanthine oxidase, pyruvate kinase, catalase, and aldolase as standards. The protein standards were mixed, applied to a column of Sephadex G-200 (2.5 × 95 cm), and eluted with the Tris·HCl buffer mentioned above. The Stokes' radius (a) so determined for AMP nucleosidase was then substituted into eq 1 in order to determine M, the molecular weight of the

$$M = \frac{6\pi\eta NaS}{1 - \bar{v}\rho} \quad (1)$$

enzyme, where N = Avogadro's number, S = sedimentation coefficient, η = viscosity of the medium, ρ = medium density, and $\bar{v}$ = partial specific volume of the protein. The partial specific volume ($\bar{v}$) of AMP nucleosidase was calculated from the amino acid composition according to the method of Cohn and Edsall (1943). These data were also used to calculate the frictional coefficient, f/f_0, according to eq 2.

$$f/f_0 = a/(3\bar{v}M/4\pi N)^{1/3} \quad (2)$$

The molecular weight was also determined in the same buffer according to the Archibald approach to equilibrium method (Trautman, 1956). The centrifuge was operated at 12,590 rpm, using schlieren optics with the phase-plate angle set at 70° and a protein concentration of 6 mg/ml. Calculations of the molecular weight were made only from the top meniscus.

Amino acid analysis was performed in a Technicon Auto-Analyzer by a modified Moore and Stein (1954) procedure outlined in the Technicon instruction manual (AAA-1, Appendix 4). Cysteine was determined as cysteic acid following performic acid oxidation according to the method of Hirs (1967), and by titration of the enzyme sulfhydryl groups with DTNB (5,5′-dithiobis(2-nitrobenzoic acid) in Tris·HCl (0.1 M, pH 8.0) containing 0.1% sodium dodecyl sulfate. Tryptophan was determined spectrophotometrically in 6 M guanidine·HCl according to the method of Edelhoch (1967).

Total carboxyl groups of AMP nucleosidase were determined in 6 M guanidine·HCl by esterification with [^{14}C]glycine ethyl ester in the presence of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (Hoare and Koshland, 1967). Minimum molecular weight of the polypeptide chains from amino acid analysis was calculated according to the method proposed by Nyman and Lindskog (1964) as modified by Black and Hogness (1969).

Polyacrylamide gel electrophoresis of the native enzyme was performed at room temperature with a current of 3 mA/tube using a Canalco Model 1200 electrophoresis apparatus (Davis, 1964). Sodium dodecyl sulfate electrophoresis was carried out at room temperature and a current of 8 mA/tube according to the method of Weber and Osborne (1969) except that 5.6-cm tubes were used. Gel electrophoresis in 9 M urea at pH 4.0 was carried out according to the method of Parish and Marchalonis (1970).

Purification of AMP Nucleosidase. Unless otherwise mentioned, *all* buffers used during the purification procedure contained EDTA (10^{-4} M), dithiothreitol (10^{-4} M) and phenylmethylsulfonyl fluoride (3×10^{-6} M). Temperatures were at 0–5° except where noted.

Initial Extract. Frozen cells (954 g) were thawed in a 30° water bath for 2 hr and suspended by mixing with 1.0 l. of 0.4 M potassium phosphate buffer (pH 7.5) containing EDTA (2×10^{-4} M), dithiothreitol (2×10^{-4} M), and PMSF (6×10^{-6} M). The suspension was passed through an Aminco French pressure cell at 15,000–25,000 psi and diluted to 3.3 l. with 0.2 M potassium phosphate buffer (pH 7.5). A 5-ml sample of the extract was centrifuged at 12,000g for 15 min and the supernatant was used to determine activity and protein.

Heat Treatment. The initial extract, placed in a 3.5-l. stainless steel beaker, was heated to 60° while stirring over a period of 30 min. The extract was maintained at 60° for 10 min and then cooled by placing the beaker in an ice bath. Supernatant (2.5 l.) was recovered after centrifugation at 12,000g for 8 hr (5°). Heating rates were not critical during this step, and similar results were obtained when the temperature was increased to 60° in as little as 2 min.

Ammonium Sulfate Fractionation. The supernatant from the heat treatment was brought to 0.37 saturation at 2° by the addition of solid ammonium sulfate over a period of 13 min followed by an additional 17-min stirring. The precipitate was

TABLE I: Purification of AMP Nucleosidase.[a]

Purification Procedure	Vol (ml)	Total Protein (mg)	Total Units (μmoles/min)	Sp Act. (μmoles/min per mg)	% Yield
Initial extract	3300	108,000	2700	0.025	100
Heat treatment	2500	32,000	2100	0.067	78
$(NH_4)_2SO_4$ fractionation	208	8,100	1500	0.19	58
Sephadex G-25	250	7,500	1500	0.21	58
First DEAE-Sephadex column	230	300	1400	4.6	51
Second DEAE-Sephadex column	57	130	1300	9.9	48
Hydroxylapatite column	7.4	60	1100	18	39
Sephadex G-200 fractionation[b]	3.2	21	690	34	34[d]
Crystals from G-200 side fractions[c]	1.1	6.7	230	34	

[a] Starting material was 954 g wet weight of *Azotobacter vinelandii* OP. [b] Fractions (inner pair of arrows, Figure 1c) containing enzyme of specific activity 34 were pooled, concentrated, and frozen for storage. [c] In this purification, fractions containing substantial activity, but specific activities less than 34, were pooled, concentrated, brought to near precipitation with $(NH_4)_2SO_4$, and crystallization induced by the addition of 1 μl of a solution containing seed crystals of AMP nucleosidase. These were collected by centrifugation, dissolved in Tris·HCl (0.1 M, pH 8.0) containing 2 mM AMP, dialyzed to remove $(NH_4)_2SO_4$, and assayed for protein and activity. [d] Per cent yield includes both fractions with specific activity of 34.

removed by centrifugation (12,000*g* for 40 min) and suspended in 150 ml of 0.1 M Tris·HCl buffer (pH 8.0) containing 0.15 M NaCl and 2 mM AMP (the Tris–NaCl–AMP buffer) to give a total volume of 208 ml.

Desalt on G-25 Sephadex. The ammonium sulfate fraction was applied to a large (4.6 × 56 cm) G-25 Sephadex column which had been preequilibrated with the Tris–NaCl–AMP buffer, and eluted with the same buffer. Those protein fractions free of ammonium sulfate, as determined by Nessler reagent, were pooled to give 250 ml.

First DEAE A-50 Sephadex Fractionation. The desalted protein solution was applied to a 4.7 × 12 cm DEAE A-50 Sephadex column equilibrated against the Tris–NaCl–AMP buffer. The protein was eluted with a 2-l. linear gradient of NaCl (0.15–0.30 M) in the same buffer. The enzyme peak appeared at 0.23 M NaCl (Figure 1a), and was diluted with the Tris–AMP buffer containing no NaCl, to reduce the NaCl concentration to 0.15 M. The solution was concentrated to 230 ml on an Amicon Diaflow pressure dialysis unit.

Second DEAE A-50 Sephadex Fractionation. The solution from the previous step was applied to a 2.5 × 50 cm DEAE A-50 Sephadex column and the column was developed using a 2-l. gradient from 0.15 to 0.28 M NaCl. Fractions of the highest activity were pooled (Figure 1b) and concentrated to 57 ml in an Amicon Diaflow concentrator.

Hydroxylapatite Fractionation. Without further adjustment of the NaCl concentration, the fraction from the second DEAE column was applied to a 1.5 × 7.5 cm column of hydroxylapatite: cellulose mixture (1.4:1.0 dry weight basis) which had been equilibrated by washing with 10 volumes of Tris–NaCl–AMP buffer. The column was eluted sequentially with 25-ml portions of the buffer containing 20, 40, 80, 160, and 320 mM K_2HPO_4 (pH 8.0). Fractions containing enzymatic activity were pooled (Figure 1c) and concentrated on an Amicon Diaflow concentrator to 7.4 ml.

Sephadex G-200 Gel Filtration. The solution from the previous step was passed through a (2.5 × 100 cm) column of Sephadex G-200 equilibrated with Tris·HCl buffer (0.1 M, pH 8.0). Those fractions containing the highest specific activities (fractions bounded by inner pair of arrows, Figure 1d) were combined and concentrated by pressure dialysis (Amicon Diaflow) to 3.2 ml.

A summary of a purification procedure is presented in Table I. Polyacrylamide gel electrophoresis of the enzyme

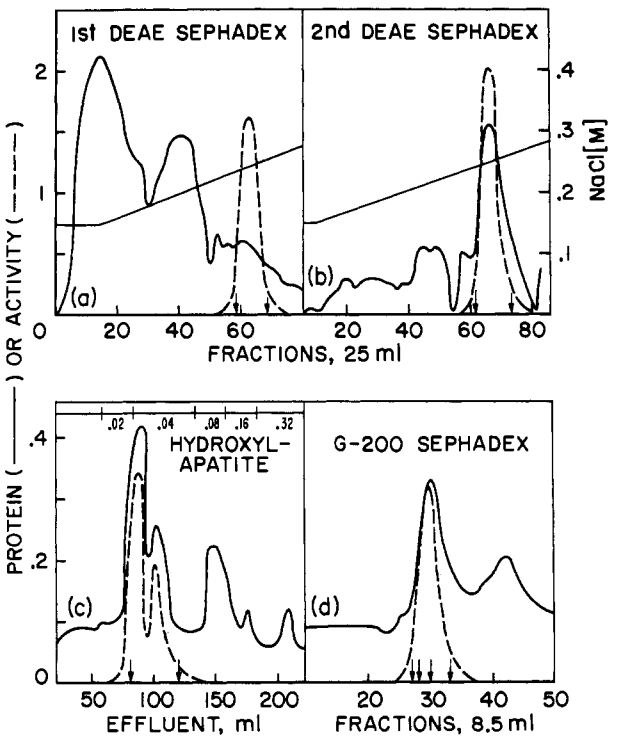

FIGURE 1: Purification of AMP nucleosidase by column chromatography. The fine lines with a positive slope in (a) and (b) represent the increase in NaCl concentration. The values at the top of (c) represent the molar concentration of K_2HPO_4 (pH 8.0) used to elute the protein. The high blank value for protein in (d) (tubes 0–23) is due to Tris interference in the Folin protein method. In all cases protein and activity were measured as described in Methods, and are expressed in arbitrary units. Other details of procedure are provided in the text.

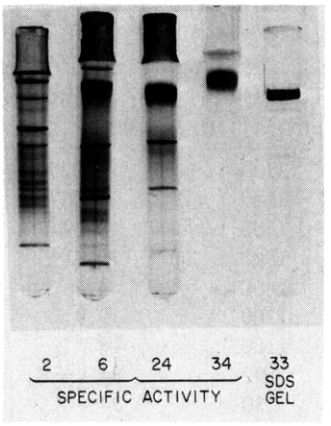

FIGURE 2: Protein electrophoresis patterns during the final stages of AMP nucleosidase purification. Amount of protein applied was 50, 150, 90, 75, and 25 μg for specific activities of 2, 6, 24, 34, and 33 μmoles per min per mg, respectively. Protein in tubes underlined by the bracket was stained with Amido-Schwarz, while the sodium dodecyl sulfate gel was stained with the more sensitive Coomassie Blue technique. No spacer gel was used in the sodium dodecyl sulfate electrophoresis.

during the final stages of purification showed an increasing concentration of a slowly migrating protein component (Figure 2). After the Sephadex G-200 fractionation this band appeared to be homogeneous, and attempts to crystallize the enzyme were made to determine if the specific activity could be further increased.

Crystallization of AMP Nucleosidase. Purified enzyme (4.6 ml; 1.7 mg/ml, specific activity of 30) in 0.1 M Tris·HCl (pH 8.0) containing 1 mM AMP was precipitated by the addition of solid ammonium sulfate to 0.75 saturation. The precipitate was collected by centrifugation at 29,000g for 7 min and extracted sequentially with 1 ml of 0.70, 0.66, 0.62, 0.58, 0.54, 0.50, 0.46, and 0.42 saturated ammonium sulfate in 0.1 M Tris·HCl–1 mM AMP (pH 8.0). Crystals were formed according to the procedure outlined by Jakoby (1968); however, the specific activities varied from 26 to 30, indicating that heterogeneous crystalline material was formed by this method. Upon standing at 4°, a second crop of crystals appeared in tubes containing 0.42 and 0.46 saturated $(NH_4)_2SO_4$, both having specific activities of 34.3, and both giving a single band in polyacrylamide gel electrophoresis. Microscopic examination (Figure 3a) revealed the crystalline structure to be hexagonal plates with a diameter of ~0.03 mm. These crystals have been used to induce crystallization using enzyme preparations with specific activities as low as 15. The resulting crystalline material always exhibited specific activities near 34 and appeared either as hexagonal plates or clusters of needles (Figure 3a,b).

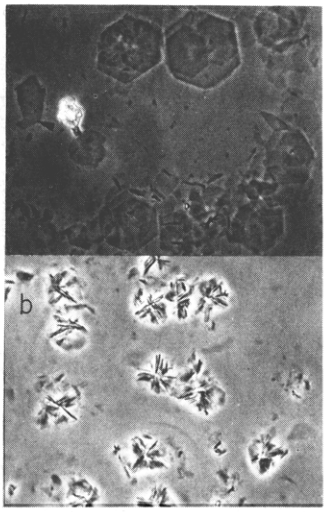

FIGURE 3: Morphology of AMP nucleosidase crystals in mother liquor. Both micrographs were taken using phase-contrast illumination with a magnification of 660 diameters. The upper illustration shows the hexagonal plates formed from slowly concentrating $(NH_4)_2SO_4$ solution. The diameter of the largest crystal is 0.03 mm. The lower illustration shows AMP nucleosidase crystals which formed as clusters of needles when seed crystals such as those shown in the upper photograph were added to an $(NH_4)_2SO_4$–enzyme solution near the protein precipitation point. Both crystalline forms had equal specific activities.

TABLE II: Amino Acid Composition of AMP Nucleosidase.[a]

Amino Acid	Molar Fraction	Residues/57,300
Glu[b]	0.1291	57.5
Leu	0.1184	55.5
Ala	0.1109	90.6
Arg	0.0834	30.6
Gly	0.0827	83.0
Val[c]	0.0827	47.8
Asp[b]	0.0813	40.5
Thr[d]	0.0556	31.6
Ser[d]	0.0507	33.4
His	0.0346	14.5
Ile[c]	0.0330	16.7
Pro	0.0312	18.4
Phe	0.0301	11.7
Tyr	0.0301	10.7
Lys	0.0175	7.8
Trp[e]	0.0119	3.7
Met	0.0102	4.5
Cys[f]	0.0063	4.1 (3.1)[g]

[a] Except where otherwise noted, values presented are the average of four determinations; hydrolysis times were 20, 21, 41, and 100 hr. [b] Values for Glu and Asp include glutamine and asparagine, respectively. [c] Average of the 41- and 100-hr determinations only. [d] Corrected for loss during hydrolysis by extrapolation to zero time. [e] Average of three spectrophotometric determinations according to the method of Edelhoch (1967), as described in Methods. [f] A single determination as cysteic acid following performic acid oxidation. [g] As determined by reaction with DTNB in 0.1% sodium dodecyl sulfate.

Stability of the Purified Enzyme. Following Sephadex G-200 the enzyme was stable for periods of at least 1 year after freezing in Dry Ice–ethanol mixtures and storing at −70°. The crystalline enzyme was also stable for periods of a least 1 year at 4°, stored in the ammonium sulfate solution from which it was crystallized.

Amino Acid Composition. The results of amino acid analysis presented in Table II, indicated that the enzyme contains low molar fractions of the aromatic amino acids, and sulfur containing amino acids, methionine and cysteine, while the ratio of acidic (Glu,Asp) to basic (Arg,His,Lys) amino acids is $\geqq 1.5$, based on amino acid and carboxyl group analysis. Carboxyl analysis revealed that less than 20% of the total

Glu + Asp residues were present as the respective amides. This may account for the relatively high affinity of the enzyme for DEAE-Sephadex, as AMP nucleosidase is one of the last proteins to be removed by increasing ionic strength (see Figure 1a). This finding is also consistent with the previous suggestion that partially purified enzyme contains a disproportionate number of negative charges, which, when uncovered in a low ionic strength environment, cause the observed disruption of subunit structure (Schramm and Hochstein, 1971).

Based on the calculated minimum subunit molecular weight of 57,300 (see Subunit Molecular Weight), the enzyme contains 4.1 cysteine (as cysteic acid) residues per subunit. Studies with DTNB, which reacts only with free sulfhydryl groups (Ellman, 1959), indicated the presence of 3.1 free sulfhydryl groups per subunit. These data indicate that few, if any, disulfide bonds are present in AMP nucleosidase, but do not preclude the possibility of intersubunit disulfide bonds. However, polyacrylamide gel electrophoresis in 9 M urea indicated that intersubunit disulfide bonds are not likely to be present in the enzyme (see Subunit Molecular Weight).

The amino acid composition was also used to calculate the partial specific volume ($\bar{v}$), according to the method of Cohn and Edsall (1943). From the data of Table II, the partial specific volume of AMP nucleosidase was determined to be 0.736.

Spectral Properties of AMP Nucleosidase. Buffered solutions of enzyme containing 6 mg of protein/ml were colorless and exhibited no absorption from 310 to 650 nm. The ultraviolet spectrum of the crystalline enzyme was determined after passage through G-200 Sephadex (1.2 × 42.5 cm) equilibrated with triethanolamine·HCl (0.05 M, pH 8.0 containing 2×10^{-4} M dithiothreitol and 10^{-3} M EDTA) to remove $(NH_4)_2SO_4$ and AMP from the enzyme solution. The enzyme exhibited a maximum absorbance at 280 nm, with $A_{280}/A_{260} = 1.88$ and $E^{1\%}_{A_{280}} = 9.73$, using the tyrosine–tryptophan absorbance of the sample as the standard for protein content (see Methods).

Molecular Weight of the Native Enzyme. Results of sedimentation velocity studies with AMP nucleosidase at protein concentrations of 1–6 mg/ml gave a $s^0_{20,w}$ value of 14.8 S, with only a slight dependence of sedimentation coefficient on protein concentration. The enzyme sedimented as a single, symmetrical boundary, and no aggregation was observed at concentrations to 6 mg/ml.

Previous results indicated that partially purified AMP nucleosidase preparations underwent a rapid dissociation reaction in the absence of protective agents (high salt or substrate) to give an enzyme form with approximately one-half the molecular weight of the native enzyme (Schramm and Hochstein, 1971). However, when a sample of purified AMP nucleosidase was dialyzed against buffer (Tris·HCl, 0.1 M, pH 8.0) free of AMP and the $s_{20,w}$ determined, there was no change in sedimentation rate, and the enzyme still sedimented as a single boundary. These results indicate that the crystalline enzyme exhibits more stable subunit interactions than the partially purified enzyme previously described.

Gel filtration studies using a number of Stokes' radius standards gave a value of 58.3 Å for the enzyme by plotting elution volume against Stokes' radius. A separate experiment in which the enzyme was passed through the column in the absence of added proteins gave the same elution position for AMP nucleosidase. These constants were used along with the calculated value of 0.736 for the partial specific volume of the enzyme as determined from amino acid analysis to give a molecular weight of 370,000 for the enzyme.

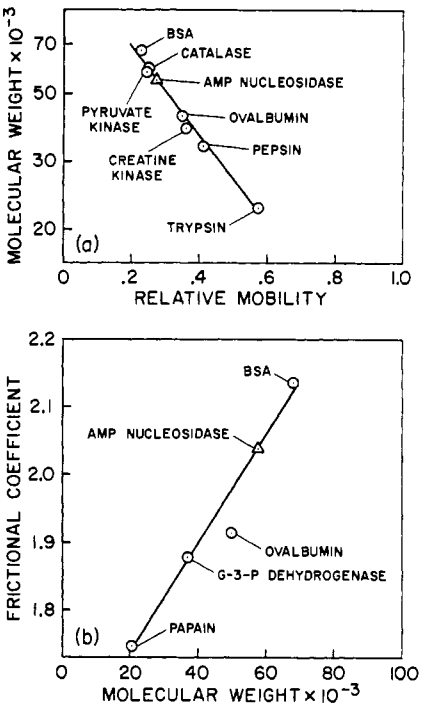

FIGURE 4: Determination of subunit molecular weight by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (a) or in 9 M urea at pH 4.0 (b). Molecular weights of standards were those used by Weber and Osborn (1969). Both lines were fitted by eye.

An alternative measure of the enzyme molecular weight was provided by the Archibald approach to equilibrium technique. Two such determinations gave molecular weights of 320,000 and 330,000, respectively, which were lower than the molecular weight of 370,000 calculated from Stokes' radius and sedimentation velocity data. The average of these values, 350,000, may be considered as a tentative molecular weight value for AMP nucleosidase.

The above physical parameters for the enzyme were used in eq 2 to determine a frictional coefficient, f/f_0, of 1.25 for AMP nucleosidase. Because the frictional coefficient is dependent both on the shape of the protein and its degree of hydration, no quantitative conclusions could be reached about the axial ratio for AMP nucleosidase. It is useful, however, to make a qualitative comparison of the frictional coefficient for AMP nucleosidase (1.25) with that of hemoglobin (1.24), which has been shown to be a compact and nearly spherical molecule by X-ray diffraction studies. On this basis, and assuming a similar degree of hydration for both proteins, it appears that AMP nucleosidase may also be nearly spherical.

Subunit Molecular Weight. The subunit molecular weight for AMP nucleosidase was determined by polyacrylamide gel electrophoresis in both sodium dodecyl sulfate and in 9 M urea (pH 4.0) and was also estimated from the minimum molecular weight derived from amino acid analysis. The result of one sodium dodecyl sulfate–polyacrylamide gel electrophoresis experiment in which AMP nucleosidase was run with seven molecular weight standards is shown in Figure 4. The data are consistent with a minimum molecular weight of 56,000. A single band of protein was observed when the gel was loaded with 5 or 50 μg of enzyme protein. In addition, molecular weight determinations were made at varying gel concentrations in 9 M urea (pH 4.0) (Parish and Marchalonis,

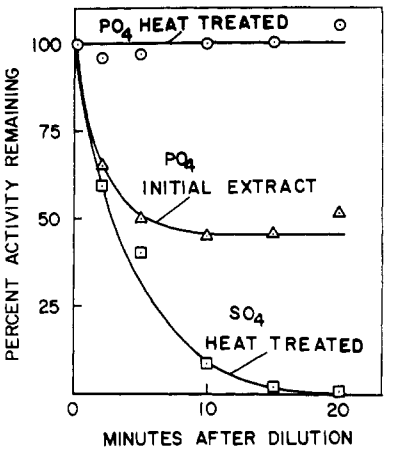

FIGURE 5: Stability of AMP nucleosidase during initial purification procedures. Enzyme stability was determined at 30° by diluting 0.02 ml of the extracts described in Table IV into 0.38 ml of Tris·HCl (0.05 M, pH 8.0) containing no other additions. Enzyme activity was determined by removing samples at the specified times and assaying in 0.25 ml of the reaction mixture described in Methods.

TABLE III: Summary of Molecular Weight Data for AMP Nucleosidase.

Technique	Calcd Mol Wt
Archibald, approach to equilibrium	325,000[a]
G-200 Stokes' radius and $s^0_{20,w}$	370,000
Polyacrylamide gel electrophoresis in sodium dodecyl sulfate	56,400[b]
Polyacrylamide gel electrophoresis in 9 M urea, pH 4.0	58,200[c]
Amino acid analysis	65,000

[a] Average of two determinations. [b] Average of four determinations, one of which is shown in Figure 6a. [c] Average of two determinations one of which is shown in Figure 6b.

1970). In the urea system, the molecular weight was determined by comparing the frictional coefficients (the degree of subunit retardation as a function of increasing polyacrylamide gel concentration) of proteins of known molecular weights with those of the unknown. In this system AMP nucleosidase exhibited a molecular weight of 58,000. Only a single sharp band appeared when the enzyme was treated with 2-mercaptoethanol prior to electrophoresis. In the absence of such treatment the enzyme ran as three distinct bands; a heavily stained band of molecular weight 58,000, and two fainter bands which were apparently of larger molecular weight. The latter bands may be aggregates of the 58,000 form resulting from the formation of intersubunit disulfide bonds. The predominance of the 58,000 molecular weight subunit form before treatment with reducing agents suggest that no intersubunit sulfhydryl linkages occur in AMP nucleosidase.

The third method of subunit molecular weight determination relied on the amino acid composition, which should be defined by integers when the numbers of amino acid residues are calculated using the correct minimum molecular weight. Using this procedure (see Methods) a subunit molecular weight of 65,000 was estimated for AMP nucleosidase.

A summary of the molecular weight data is presented in Table III. There is good agreement between the subunit molecular weights obtained by the two independent polyacrylamide gel techniques, while the minimum molecular weight obtained from amino acid composition is significantly larger. The values obtained from polyacrylamide gel analysis are considered to be closer to the actual subunit molecular weight and an average gives a subunit molecular weight of 57,300. Using the average of 350,000 for the molecular weight of the native enzyme, the data are in agreement with six of the 57,300 molecular weight subunits per enzyme molecule. However the data are insufficiently precise to rule out other possibilities. Since recent studies have shown enzymes which consist of both five (*e.g.*, arginine decarboxylase, Boeker and Snell, 1968) and six (*e.g.*, inorganic pyrophosphatase, Hall and Josse, 1967; *S*-aminolevulinate dehydratase, Doyle, 1971) subunits, additional data will be required to verify a hexameric structure.

In three independent systems which are potentially capable of detecting differences in subunit structure, namely, polyacrylamide in sodium dodecy sulfate and in 9 M urea, and ultracentrifugation in sodium dodecyl sulfate–dithrothreitol, no evidence of nonidentical subunits was observed. Thus it is possible that AMP nucleosidase may be composed of identical subunits. However, the results do not preclude the possibility of similar but distinct subunits in AMP nucleosidase. Experiments are now in progress to define the subunit composition of the enzyme in a more precise manner.

Stabilization of AMP Nucleosidase. Crystalline AMP nucleosidase showed no dissociation when diluted into low ionic strength buffers or buffers free of AMP. This result differed from the results previously obtained with partially purified AMP nucleosidase (Schramm and Hochstein, 1971) and from the results obtained by Ogasawara *et al.* (1970) with a homogeneous preparation of the enzyme. It was therefore of interest to determine which step in the present purification procedure caused this change in the enzyme. Starting with frozen *A. vinelandii*, the enzyme was purified in parallel according to both purification procedures. Samples were tested for stability in low ionic strength buffer at each step of the purification (Figure 5) and the results are summarized in Table IV. The purification steps were similar in both procedures, except that 0.25 M K_2HPO_4 (pH 7.5) was used as the stabilizing agent in the present purification, while 0.25 M K_2SO_4 was used previously. It is apparent that partial stabilization of the enzyme prepared in K_2HPO_4 occurred during the preparation of initial extract and was completed during the heating step, when the extract was heated to 60° for 10 min. As stabilization occurred in the presence of phosphate but not sulfate, it appeared that phosphate assisted in the formation of the stable enzyme, or alternatively, that sulfate acted to inhibit the formation of the stable enzyme complex. In addition, similar stabilization occurred when 2 mM AMP replaced K_2SO_4.

These observations on the stability of AMP nucleosidase make it quite clear why previous preparations of the enzyme (Yoshino, 1970; Schramm and Hochstein, 1971) were unstable following purification, as both procedures employed a heat step in 0.25 M K_2SO_4. In addition, the purification procedure of Yoshino (1970) used distilled water for the disruption of the bacterial cells, as well as steps in low ionic strength buffer. Such treatment probably accounts for the observed molecular weight of 240,000 for the enzyme (which dissociated to 120,000 in low ionic strength buffer) and the low specific activity (~18) observed for that preparation of AMP nucleosidase (Yoshino, 1970; Ogasawara *et al.*, 1970). At the present time, the mechanism of the conversion from an unstable to a

TABLE IV: Comparison of AMP Nucleosidase Stability[a] during Purification in K_2SO_4 (0.25 M)[b] or K_2HPO_4 (0.2 M, pH 7.5).

	Purified in K_2SO_4					Purified in K_2HPO_4				
Step	Vol (ml)	Protein (mg/ml)	Sp Act. (μmole/min per mg)	Total Units	Stability %	Vol (ml)	Protein (mg/ml)	Sp Act. (μmole/min per mg)	Total Units	Stability (%)
Initial extract	42	26	0.020	23	16	31	34	0.025	26	45
Heat treatment (60° for 10 min)	72	4	0.043	13	1	22	13	0.060	17	100

[a] Stability is expressed as the percent activity remaining after 20 min at 30° following a 1:20 dilution into Tris·HCl buffer (0.05 M, pH 8.0). [b] K_2SO_4 solution was buffered with Tris (0.1 M, pH 8.0) containing EDTA (10^{-4} M), dithiothreitol (10^{-4} M), and PMSF (3×10^{-6} M).

stable enzyme form cannot be fully explained, and additional experiments will be required to elucidate the mechanism of conversion.

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