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Purification, Subunit Structure, and Amino Acid Composition of Avian Erythrocyte Adenosine Monophosphate Deaminase†

Walter C. Kruckeberg,‡ Shelia Lemley, and Oscar P. Chilson*

ABSTRACT: Adenosine monophosphate deaminase has been purified over 6000-fold from chicken erythrocytes to a specific activity of 976 units/mg in 25% yield. The procedure involves ammonium sulfate fractionation, chromatography on DE-52 cellulose, further ammonium sulfate fractionation, and chromatography on phosphocellulose. Electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate showed that the subunit size of the erythrocyte isozyme corresponds to a molecular weight identical to that of the breast muscle isozyme (i.e., 69 000). Antibody directed against the purified erythrocyte AMP deaminase showed a single precipitin band on Ouchterlony plates when tested against the RBC enzyme but did not cross-react with muscle AMP deaminase. Cosedimentation of native muscle and erythrocyte AMP deaminases on sucrose velocity gradients showed that the molecular weights of these isozymes are virtually identical. The purified erythrocyte isozyme is highly specific for 5′-AMP; the apparent rates of deamination of adenosine, 5′-ADP, and 5′-ATP were less than 1% of the rates observed with 5′-AMP. The substrate concentration required to attain 50% V_{max} ($S_{0.5}$) in the absence of potassium ion is 4.5 mM. Kinetic studies performed in the presence and absence of potassium ion yielded data that are consistent with theoretical curves for a four-subunit allosteric model, and K_1 (the dissociation constant for the R conformational state) estimated from these data is 1.8×10^{-3} M 5′-AMP. V_{max} is unchanged over the range of [KCl] from 0 to 200 mM. Substrate saturation data obtained at various concentrations of phytic acid, a potent allosteric inhibitor of the erythrocyte deaminase, indicate that phytate binds most readily to the conformation having the lower affinity for substrate. Phytate inhibition is reversed at saturating levels of 5′-AMP and is also sharply diminished in the presence of KCl; e.g., when assayed at 2 mM 5′-AMP the concentrations of phytate required for 50% inhibition of enzymatic activity in the absence and presence of 150 mM KCl were 1 μM and 4 mM, respectively. The characteristics of phytate inhibition indicated that the mechanism of inhibition is not due to chelation of Zn(II). The amino acid composition of chicken erythrocyte AMP deaminase is similar but not identical to the isozyme from avian breast muscle. The subunit molecular weight of the red blood cell isozyme based on amino acid composition is in good agreement with the subunit molecular weight determined by sodium dodecyl sulfate–gel electrophoresis.

AMP deaminase is an allosteric enzyme that exists in several molecular forms. Catalytic and immunological differences (Henry and Chilson, 1973), as well as developmental studies (Sammons and Chilson, 1977; Kaletha and Zydowo, 1971), provide indirect evidence for functional differences among these isozymes and suggest that they are regulated differently. However, the molecular bases for the AMP deaminase isozymes are not known. A major reason for the lack of structural detail regarding these isozymes is that purification to homogeneity, in quantities sufficient for molecular studies, is made difficult by the fact that AMP deaminase activity is generally very low in nonmuscle tissues (Conway and Cooke, 1939; Lowenstein, 1972). Until recently, skeletal muscle was the only tissue from which highly purified preparations had been ob-

† From the Department of Biology, Division of Biological and Biomedical Sciences, Washington University, St. Louis, Missouri 63130. *Received April 12, 1978.* This investigation was supported by the National Science Foundation (NSF-GB-40710) and the National Institutes of Health (AM 16741).
‡ Present address: Department of Human Genetics, University of Michigan, Ann Arbor, Mich.

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tained. The enzyme has now been isolated and purified from human erythrocytes, and some of its kinetic and molecular properties were described (Yun and Suelter, 1978). Inasmuch as AMP deaminase activity is exceptionally high in chicken erythrocytes (Henry and Chilson, 1973; Kruckeberg and Chilson, 1973), we have focused our efforts on the isolation and characterization of this isozyme.

In the study to be reported here, we have (1) purified AMP deaminase from adult chicken erythrocytes, (2) determined its subunit molecular weight and amino acid composition, and (3) analyzed certain aspects of its kinetic properties in terms of an allosteric model and the possible role of phytic acid in the regulation of enzymatic activity.

Experimental Procedure

Materials

Sodium dodecyl sulfate (Sequenal grade) was purchased from Pierce Chemical Co. Acrylamide, *N,N'*-methylenebisacrylamide, 2-mercaptoethanol, and *N,N,N',N'*-tetramethylethylenediamine were obtained from Eastman and Bio-Rad. Adenosine 5'-monophosphate (free acid) was from Miles Laboratories, Inc. Dithiothreitol, heparin, adenosine 5'-diphosphate, adenosine 5'-triphosphate, sodium phytate, DEAE[1]-cellulose (medium grade, 0.88 mequiv/g), Trizma base, 2-(*N*-morpholino)ethanesulfonic acid (Mes), sucrose, and Coomassie blue were obtained from Sigma Chemical Co. Phosphocellulose, medium grade (capacity 0.5 mequiv/g), and "selectacel" phosphate (1.09 mequiv/g) were obtained from Sigma and the Brown Co., respectively. Whatman DE-52 diethylaminoethylcellulose was obtained from Reeve Angel. Bio-Gel A5m (200–400 mesh) was from Bio-Rad. Mercaptoethanesulfonic acid (Sequenal grade) was from Pierce Chemical Co. Water used throughout was passed through a bed of activated charcoal, followed by a mixed ion-exchange resin (both supplied by Continental Deionized Water Service). Other reagents were analytical grade and were used without further purification.

Methods

Preparation of Lysate. Red blood cells from adult chickens were obtained from St. Louis Serum Co. or Pel-Freeze Biologicals, Inc. Whole blood was collected into and washed with heparinized citrate–saline solution (10 mg of heparin + 40 g of sodium citrate + 8.5 g of sodium chloride/L). These washed cell suspensions were shipped on ice from the collection point and were normally received within 24 to 36 h after collection. Upon arrival, the cells were washed twice as described above and then were suspended in 2 volumes of citrate saline (without heparin), and the suspension was frozen at −20 °C. Upon thawing, the slurry was centrifuged at 8000*g* (4 °C), the sediment was discarded, and the AMP deaminase was purified from the crude lysate supernatant as described below. (Attempts to fractionate lysates prepared in physiological saline with ammonium sulfate, as described below, led to the formation of a gel which could not be used for purification of enzyme.)

Assay of Enzymatic Activity. Enzymatic activity was routinely determined by the discontinuous assay of the initial rate of ammonia production at 37 °C, as described by Henry and Chilson (1973). One unit of enzymatic activity is defined as the amount of enzyme required to catalyze the deamination of 1 μmol of 5'-AMP/min when assayed as described above, at concentrations of KCl and 5'-AMP which were shown to be saturating. For certain experiments (specified below), the more convenient continuous assay of Kalckar (1947) was used. A semiquantitative enzyme assay was routinely used when column eluates were screened for enzymatic activity. Aliquots (0.5–0.1 mL) of the fractions to be tested were combined with 0.3 mL of 40 mM 5'-AMP in 50 mM Tris–succinate + 0.1 M KCl (pH 6.5). After incubation for 20–30 min at 37 °C, 4.5 mL of water and 0.5 mL of Nessler's reagent (Bradstreet, 1965) were added. The resulting color was noted visually or the absorbance at 420 nm was read.

Protein Determinations. As specified below, protein was routinely determined either by the method of Lowry et al. (1951) or the microbiuret procedure of Munkres and Richards (1965); however, protein in column eluates was estimated by the procedure of Warburg and Christian (1957).

Conductivity Measurements. The relative ionic strengths of buffers, dialysates, and column eluates were assessed by measuring the conductivity at 25 °C with a Radiometer conductivity meter, Model CDM2e, using a Model CDC 114 conductivity cell.

Analytical Polyacrylamide Gel Electrophoresis. Electrophoresis in the absence of detergent was performed essentially as described by Ornstein (1964) and Davis (1964). Optimal results were obtained when enzyme samples (1 mg of protein/mL) were dialyzed against 64 mM Tris–phosphate (pH 6.9) buffer prior to electrophoresis; furthermore, it was necessary to reduce the current to no more than 1 mA/gel until the tracking dye had entered the running gel.

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate was performed as described by Fairbanks et al. (1971). The gels were stained for protein either with Fast green or Coomassie blue, depending upon the amount of protein applied to the gels (Weber et al., 1972). Proteins consisting of polypeptide chains of known size, as reported by Darnall and Klotz (1975), were used as standards.

Immunological Procedures. Rabbit antisera directed against two preparations of erythrocyte AMP deaminase (specific activity 950 and 976 units/mg) were obtained as follows: 5.0 mg of lyophilized enzyme in 0.5 mL of phosphate-buffered saline (8.8 g of NaCl + 7.0 g of $NaH_2PO_4 \cdot H_2O$/L) was emulsified with 0.5 mL of complete Freund's adjuvant and injected at multiple sites in the neck region. A secondary response was elicited by injection of 1-mg amounts of lyophilized enzyme in 1.0 mL of phosphate-buffered saline in the marginal ear vein on days 32, 34, 37, and 39 following the primary injection. The antiserum used in these studies was obtained from blood collected from the marginal ear vein on day 43 following the primary injection. Double-diffusion (Ouchterlony) tests were performed as described by Selig and Chilson (1969).

Sedimentation in Sucrose Gradients. Linear gradients (5 mL, 5–20% sucrose) were prepared in buffer containing 0.5 M KCl + 50 mM Tris–succinate + 0.1% NaN_3 + 5 mM 2-mercaptoethanol (pH 6.5), essentially as described by Martin and Ames (1961). Samples were dialyzed for 2 h (4 °C) against the above buffer prior to layering 200-μL aliquots, containing approximately 450 μg of chicken heart lactate dehydrogenase + 450 μg of erythrocyte AMP deaminase ± 450 μg of chicken breast muscle AMP deaminase, on the gradients. Centrifugation was for 19 h at a RCF of 71 440*g* (5 °C) in a Spinco Model L ultracentrifuge, using an SW 39 rotor. Gradients were fractionated (ISCO fractionator 0.6 mL/min; 3-drop fractions) and enzymatic activities were determined

[1] Abbreviations used are: DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

by the continuous spectrophotometric assay indicated above.

Amino Acid Analysis. Analyses were performed with a Beckmann Model 120C amino acid analyzer according to the two-column methodology of Moore et al. (1958). Samples were hydrolyzed with 6 N HCl or 3 N mercaptoethanesulfonic acid (Penke et al., 1974) in sealed tubes under reduced pressure at 110 °C for 24, 48, and 72 h. Assessment of mechanical losses was made on the basis of recoveries of the internal standards norleucine and leucine as described by Walsh and Brown (1962). The possible presence of glucosamine and galactosamine was determined after hydrolysis in 4 N HCl in sealed tubes under reduced pressure for 4 h at 110 °C (Keefer and Bradshaw, 1977). Glucosamine and galactosamine were tentatively identified on these chromatograms by comparison with elution times observed when authentic samples of these amino sugars were chromatographed under identical conditions. In those cases where moisture content of lyophilized protein samples was determined, samples were dried to constant weight by heating for 24–72 h at 110 °C under reduced pressure in a heated vacuum desiccator.

Analysis of Kinetic Data. Most published data regarding the allosteric properties of AMP deaminase (e.g., Henry and Chilson, 1973; Yoshino et al., 1976; Smiley et al., 1967; Yun and Suelter, 1978) have been analyzed according to the Hill equation. This approach often generates low, nonintegral values of the interaction coefficient, n, which are not quantitatively related, in a simple way, to the demonstrated quaternary structure of the enzyme. Therefore, we chose to analyze the kinetic data obtained during these studies according to the concerted transition of Monod et al. (1965) using the equation derived by Frieden (1967):

$$v/V_{max} = \frac{(1+\alpha)^{n-1} + Lc\alpha(1+c\alpha)^{n-1}}{(1+\alpha)^n + L(1+c\alpha)^n} \quad (1)$$

The term L is the equilibrium constant for the T to R transition, where T is defined as the conformational state which has the lowest affinity for ligand and R as the conformational state having the highest affinity for ligand; c is the ratio of the substrate dissociation constants of the R and T states; i.e., c is a measure of the difference in the ability of the two states to bind the ligand; n is the number of substrate binding sites per molecule of protein; K_1 is the dissociation constant for the R conformational state; and α is the reduced substrate concentration (i.e., initial substrate concentration divided by K_1). Experimental data were plotted as v/[5′-AMP] vs. v, with v expressed as a fraction of $V_{max} = 1.0$. Certain parameters were determined by inspection, others by curve fitting. The limiting slope of the experimental curves at high values of v is equal to $-1/K_1$. In order to simplify analysis of the data, a Wang 700 series calculator was programmed so that values of L, c, n, and α could be entered to generate values of v/α and v, which were plotted on the same figures.

A completely linear plot of v/[5′-AMP] vs. v over the entire substrate saturation range indicates hyperbolic kinetics, characteristic of adherence to Michaelis–Menten behavior. In general, addition of an activator is correlated with a decrease in L and a v/[5′-AMP] vs. v plot which approaches linearity, while inhibitors cause an increase in L and nonlinear plots.

Results and Discussion

Purification of Enzyme

Step I: First Ammonium Sulfate Fractionation. Enzyme was isolated from crude lysate supernatants (prepared as described above). All preparative procedures were performed at 0–4 °C. An equal volume of saturated ammonium sulfate was added dropwise with stirring to the crude lysate supernatant. (In order to obtain good recoveries of enzymatic activity at this step, it is essential that the pH of the saturated ammonium sulfate be adjusted to pH 6.4 with 58% NH_4OH. This adjustment was made at room temperature and the saturated ammonium sulfate solution was chilled to 0–4 °C before use; adjustment of pH was not necessary at later stages of purification.) The viscosity of the 50% saturated protein suspension was reduced by the addition of an equal volume of 50% saturated ammonium sulfate. The suspension was then centrifuged at 10 000g for 20 min, at 0–4 °C. The pellet was washed with 50% saturated ammonium sulfate; the washed pellet was dissolved in a minimal volume of KCl–PO_4 buffer (90 mM potassium phosphate + 180 mM KCl, pH 6.5). The sample was then dialyzed overnight against 1 L of KCl–PO_4 buffer containing 1 mM 2-mercaptoethanol. The dialyzed sample was centrifuged as described above, the inactive pellet was discarded, and the sample was stored on ice until a total of 6.4 L of lysate supernatant had been processed to this stage (fraction I).

Step II: Second Ammonium Sulfate Fractionation. Fraction I was diluted with KCl–PO_4 buffer to an activity of 45 units/mL, and solid ammonium sulfate was added to 50% saturation (313 g/L); after standing on ice for 30 min, the suspension centrifuged (10 000g, 20 min) and the supernatant discarded. The pellet was dissolved in a minimum volume of KCl–PO_4 buffer. After overnight dialysis against the same buffer, the sample was centrifuged as described above. The pellet was discarded and the dark-yellow supernatant (fraction II) was treated as described below. Although there was little change in specific activity during this step, virtually all remaining hemoglobin, which interferes seriously with subsequent attempts to purify the enzyme, was removed. The sample was stored at −20 °C until processed as described below.

Step III: Chromatography on DE-52 Cellulose. Fraction II was diluted twofold with KCl–PO_4 buffer and dialyzed 20–26 h against two 4-L batches of 9 mM Tris–succinate + 10 mM KCl (pH 6.5)—or until the conductivity of the sample was 2.54 mmho. The sample was centrifuged as described above and the supernatant (2250 mL; 5.42 mg of protein/mL) applied to a column (7.5 × 65 cm) of DE-52 cellulose which had been equilibrated with dialysis buffer. The flow rate was maintained at 200 mL/h with a peristaltic pump. After all the sample had been applied, the column was washed with approximately 2 column volumes of the same buffer to remove unadsorbed protein. A linear gradient consisting of 3.0 L of 9 mM Tris–succinate + 10 mM KCl + 1 mM 2-mercaptoethanol (pH 6.5) and 3.0 L of 100 mM Tris–succinate + 1.0 M KCl + 5 mM 2-mercaptoethanol (pH 6.5) was applied. Fractions of 15 mL were collected at a flow rate of 200 mL/h and were assayed for protein and enzymatic activity. The enzymatic activity began to elute at a conductivity of 2.86 mmho. The pooled fractions (fraction III) were concentrated by dialysis for several hours against a 10-L bath of saturated ammonium sulfate and centrifuged as described above. The supernatant, having negligible enzymatic activity as assessed by the decrease in absorbance at 265 nm at a substrate concentration of 100 μM, was discarded. The pellet was dissolved in, and dialyzed overnight against, 0.2 M KCl + 0.1 M potassium phosphate + 1 mM 2-mercaptoethanol (pH 6.5).

Step IV: Third Ammonium Sulfate Fractionation. The dialyzed sample was diluted to 249 units/mL with dialysis buffer and solid ammonium sulfate was added to 35% saturation (209 g/L). After standing on ice for 30 min, the sample was centrifuged (16 000g for 20 min) and the supernatant

TABLE I: Summary of Purification of AMP Deaminase from Chicken Erythrocytes.[c]

step[a]	vol (mL)	protein (mg)	act. (units)	sp act. (units/mg)	yield (%)
lysate supernat.	6420	693 360	88 403	0.13	100
I	890	16 109	77 179	4.79	87.3
II	1100	11 935	73 590	6.16	83.2
III[b]	273	2 800	43 741	15.62	49.5
IV	24	433	45 696	105.6	51.7
V	6	22.14	21 627	976	24.5

[a] For a description of steps I–V, see Results and Discussion. [b] A total of 65 564 units was applied to the DE -52 cellulose column. [c] The yields are given for approximately 3 L of washed red blood cells.

made 45% saturated by the addition of solid ammonium sulfate (62 g/L). The suspension was centrifuged as described above, and the pellet was dissolved in a minimal volume of KCl–PO_4 buffer containing 1 mM 2-mercaptoethanol. The fraction collected between 35 and 45% saturation (IV) was dialyzed overnight against the same buffer.

Step V: Chromatography on Cellulose Phosphate. Fraction IV was centrifuged as described above, the pellet discarded, and the supernatant applied to a column (5.0 × 60 cm) of cellulose phosphate which had been equilibrated with the dialysis buffer. The column was washed with approximately 2 column volumes of buffer and a linear gradient, consisting of a starting buffer of 1250 mL of 0.2 M KCl + 0.1 M potassium phosphate (pH 6.5) + 1 mM 2-mercaptoethanol, and a limiting buffer of 1250 mL of 1.0 M KCl + 0.1 M potassium phosphate (pH 6.5) + 5 mM 2-mercaptoethanol was applied. The flow rate was maintained at approximately 70 mL/h. Fractions (10 mL) were assayed for enzymatic activity and protein as described above. Those fractions having appreciable enzymatic activity corresponded to a single protein peak and were pooled and concentrated on an Amicon PM 30 membrane (fraction V). The enzyme was routinely stored on ice in the presence of 3 mM 2-mercaptoethanol and 0.1% sodium azide. Table I summarizes the results of a typical preparation.

Most of the kinetic studies described in this communication were performed with preparations of enzyme purified approximately 2500-fold to a specific activity of 450–500 units/mg. The procedure involved adsorption of deaminase on DEAE-cellulose (Haut et al., 1964; Hennessey et al., 1962), chromatography on phosphocellulose, and gel-filtration chromatography on Bio-Gel A5m. The procedure described in detail above gave better yields and was the more reproducible. Enzyme preparations having specific activities of 450 units/mg or greater, purified by either method, exhibited similar catalytic properties, and the major protein band in these preparations showed the same electrophoretic mobility in polyacrylamide gels containing sodium dodecyl sulfate (vide infra).

AMP deaminase from chicken breast muscle was purified as described previously by Selig and Chilson (1969).

Enzyme Properties

Electrophoresis. The pattern obtained when AMP deaminase (freshly isolated; specific activity 916 units/mg) was electrophoresed in polyacrylamide gels in the absence of detergent is illustrated in Figure 1 (gel A). The faint protein band near the top of the gel is believed to be polymerized AMP deaminase, because of its electrophoretic behavior in gels of decreasing pore size and the fact that slices obtained from this region of the gels showed AMP deaminase activity (data not shown). Furthermore, after storage at 4 °C for several days, virtually all the protein migrated at the position of this minor band.

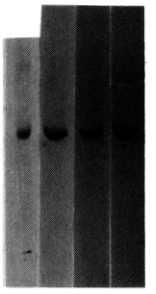

FIGURE 1: Electrophoresis of AMP deaminase in polyacrylamide gels in the absence and presence of detergent. (A) Erythrocyte AMP deaminase (specific activity 976 units/mg): Electrophoresis was in 8% gels, without detergent; conditions for electrophoresis are described under Experimental Procedure. (B) Electrophoretic pattern of erythrocyte AMP deaminase (specific activity 724 units/mg) in the presence of sodium dodecyl sulfate. Sample was treated with sodium dodecyl sulfate in the presence of dithiothreitol, electrophoresed in polyacrylamide gels containing 0.1% sodium dodecyl sulfate, and stained for protein as referenced under Experimental Procedure. (C) Same as B, except the sample was muscle adenylate deaminase (specific activity 1000 units/mg). (D) Same as B, except erythrocyte and muscle enzymes were combined.

The electrophoretic pattern observed when a preparation of erythrocyte AMP deaminase (specific activity 724 units/mg) was electrophoresed in polyacrylamide gels containing 0.1% sodium dodecyl sulfate is shown in Figure 1 (gel B). Although the preparation used for this experiment was not quite homogeneous, it contained only one major protein band, and, as this figure shows, electrophoresis of a mixture of the avian muscle and erythrocyte isozymes shows that the subunit sizes of these enzymes are indistinguishable in this electrophoretic system (gel D). Since the subunit molecular weight of breast muscle AMP deaminase is 69 000 (Boosman and Chilson, 1976), this observation shows that the subunit size of erythrocyte AMP deaminase is also 69 000. This observation is in agreement with a preliminary report from this laboratory (Kruckeberg and Chilson, 1976), as well as with the results of a recent study of the AMP deaminase from human red blood cells by Yun and Suelter (1978).

Immunological Characteristics. The chicken erythrocyte AMP deaminase is immunologically distinct from the chicken breast muscle deaminase, as tested by double-diffusion (Ouchterlony) analysis. *Antierythrocyte* AMP deaminase antisera exhibited a single, sharp precipitin band when tested against the erythrocyte enzyme. However, when this antiserum was similarly tested against chicken muscle AMP deaminase, precipitin bands were not observed. Typical results are presented in Figure 2; by this test, the interaction between muscle AMP deaminase and antibodies directed against the red blood cell isozyme is negligible. Similar studies using antibodies directed against *muscle* AMP deaminase indicated comparable strict specificity for the *muscle* isozyme (data not shown). Each

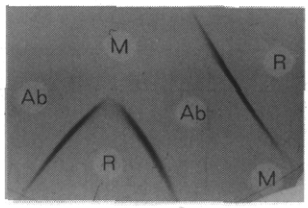

FIGURE 2: Specificity of rabbit anti-RBC AMP deaminase. Wells designated M contained purified avian breast muscle AMP deaminase, and wells designated R contained purified avian erythrocyte AMP deaminase. Wells designated Ab contained undiluted rabbit antiserum directed against purified erythrocyte AMP deaminase.

antigen and antibody was tested over a wide range of concentrations. In typical experiments, purified erythrocyte (or muscle) enzyme (0.65–1.34 mg/mL) was placed in the center well, and antiserum was placed in six to eight peripheral wells, beginning with undiluted antiserum and continuing with serial double dilutions over a 32- to 128-fold range. Comparable experiments were performed with antisera from two rabbits which had been immunized with two different preparations of purified red blood cell deaminase. Parallel double-diffusion plates were set up in which muscle and erythrocyte deaminases were tested against whole IgG isolated from sera obtained from several different rabbits which had been immunized with purified breast muscle enzyme.

Sedimentation in Sucrose Gradients. The sedimentation patterns, on velocity gradient centrifugation, of erythrocyte AMP deaminase and a mixture of the muscle and erythrocyte isozymes are compared in Figure 3 (panels A and B, respectively). Relative to the internal standard, lactate dehydrogenase, the fractions containing maximum AMP deaminase activity were identical. These results indicate that the molecular weight of adult avian erythrocyte AMP deaminase is virtually identical to that of the muscle isozyme (i.e., 276 000; Boosman and Chilson, 1976).

Substrate Specificity. Like other nonbacterial deaminases, erythrocyte AMP deaminase is highly specific for 5′-AMP. The rate of ammonia production observed at saturating levels of 5′-dAMP (expressed as a percentage of the V_{max} observed with 5′-AMP) remained approximately 5% throughout 6000-fold purification. Thus, it is probable that the hydrolytic deamination of both 5′-AMP and 5′-dAMP is catalyzed by a single enzyme. The apparent rates of deamination of adenosine, 5′-ADP, and 5′-ATP were less than 1% (data not shown).

Kinetic Properties. The apparent affinity for 5′-AMP exhibited by the chicken erythrocyte enzyme increased as the enzyme was purified. The $S_{0.5}$ for 5′-AMP (4.5 mM) of the highly purified AMP deaminase described here was several fold lower than the $S_{0.5}$ values of less purified preparations described previously (specific activities of 120–140 units/mg; Kawamura, 1972; Henry and Chilson, 1973). Therefore, we have reexamined certain aspects of the regulatory properties of the highly purified enzyme and have correlated these allosteric parameters with the demonstrated quaternary structure.

In the absence of potassium chloride the kinetic behavior of the highly purified enzyme with respect to 5′-AMP showed marked cooperative interaction, but in the presence of potassium chloride cooperativity was sharply decreased, as shown by the approach to linearity of plots of $v/[5'\text{-AMP}]$ vs. v at high velocity. This characteristic simplified the determination of the slope, thus facilitating estimation of K_1 (the dissociation constant for the R conformational state). However, cooperativity was not completely eliminated by KCl, as was indicated by deviation from linearity at low substrate concentration. Plots

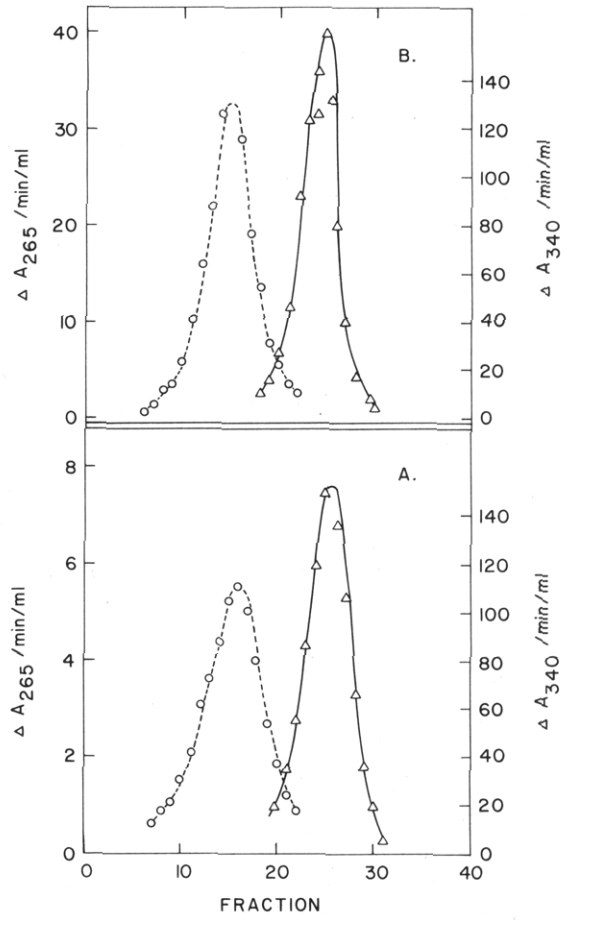

FIGURE 3: Sedimentation of erythrocyte and muscle AMP deaminases on sucrose gradients: (A) mixture of lactate dehydrogenase (○) and red blood cell AMP deaminase (△); (B) mixture of lactate dehydrogenase (○) and AMP deaminases (△) from red blood cells and muscle. Centrifugation was from left to right under conditions described under Experimental Procedure.

of $v/[5'\text{-AMP}]$ vs. v, based on the measurement of initial velocities at various levels of potassium chloride, are shown in Figure 4A; solid lines represent theoretical curves drawn according to eq 1, assuming the values of n, c, and L given in the legend.

Comparison of the experimental data with the theoretical curves shows that both in the absence and presence of potassium chloride the data are consistent with theoretical curves for a four-subunit model (i.e., $n = 4$) in which c (ratio of the dissociation constants for the R and T states) is greater than 0 but less than 0.1. The value of L (allosteric or equilibrium constant for the R ⇌ T transition) decreased from 355 (at 0 mM KCl) to 22 (at 1 mM KCl), to 3.6 (at 3 mM KCl), to 0.65 (at 10 mM KCl), while c remains constant at 0.06. The theoretical curves for n values of 2 or 8, or odd numbers (not shown), were not in agreement with the experimental data. Thus, the kinetic data are consistent with a model in which there is one binding site for 5′-AMP per enzyme subunit.

When the concentration of potassium chloride in the assay mixture was increased from 10 to 100 mM, data (not shown) very similar to those shown for 10 mM potassium chloride were obtained. Addition of 5′-ATP (8.1 mM, without potassium chloride) yielded similar data. However, when 200 mM KCl was added (minus ATP), the data fit a theoretical curve having a larger L value, indicating increased cooperativity and inhibition of enzymatic activity at high levels of KCl. Thus, there was an approach to hyperbolic kinetics in the presence of po-

tassium ion concentrations equal to or greater than 10 mM but less than 200 mM, as well as when ATP was added at 8.1 mM (in the absence of potassium chloride). $S_{0.5}$ values estimated from plots of v vs. [5′-AMP] in the presence and absence of ATP were 1.6 and 4.5 mM, respectively (data not shown). Apparent K_m values for 5′-AMP *approximated* from the double-reciprocal plots of the data were: 1.4 (10 mM KCl), 1.7 (30 mM KCl), 1.5 (100 mM KCl), and 1.6 mM (8.1 mM ATP).

Inhibition of AMP Deaminase by Phytic Acid. The major organic phosphate in mammalian erythrocytes, 2,3-diphosphoglyceric acid (Rapoport and Guest, 1941), is an allosteric modifier of mammalian red blood cell AMP deaminase (Askari and Rao, 1968; Lian and Harkness, 1974). In erythrocytes of mature birds, 2,3-diphosphoglyceric acid is replaced by phytic acid as the major organic phosphate (Rapoport and Guest, 1941; Isaacks et al., 1976). The allosteric effects of inorganic phosphate and nucleotides are generally reversed by potassium ion. Although allosteric inhibition of avian erythrocyte AMP deaminase has been demonstrated (Yoshino et al., 1976), the effect of potassium ion on phytate inhibition of this isozyme has not been described.

Substrate saturation curves were generated for 0, 5, and 20 μM phytic acid, at a constant concentration of KCl (30 mM). The experimental data were analyzed as described above and are summarized in Figure 4B. Solid lines in this figure represent theoretical curves generated using eq 1, assuming the values of L, c, n, and K_1 given in the figure legend. The data fit a model in which $n = 4$ and c is less than 0.1 but greater than 0. That phytic acid is an allosteric inhibitor of purified erythrocyte AMP deaminase is clearly indicated by the large increase in L (equilibrium constant for the $R \rightleftharpoons T$ transition) from 3.3 (0 phytate) to 16 (5 μM phytate) to 125 (20 μM phytate).

The effect of phytic acid is primarily, if not exclusively, on apparent substrate binding, as shown by the increased cooperativity (larger L), with approach to the same V_{max} at high 5-AMP; i.e., high substrate concentration reverses phytate inhibition. Although the experimental data are consistent with certain aspects of the allosteric model proposed by Monod et al. (1965), in order to determine the number of binding sites for phytate, L should vary as the fourth power of inhibitor concentration ($n = 4$). It is clear (see Figure 4B) that this is not the case. The data are, however, consistent with the hypothesis that phytate binds most readily to the form of the enzyme which is least active, i.e., that molecular species having the lower affinity for substrate.

Further evidence in support of this hypothesis was obtained from examination of the effect of potassium ion on inhibition by phytate. When assayed at subsaturating substrate concentration (i.e., 2 mM 5′-AMP), in the presence and absence of 150 mM potassium chloride, the concentrations of phytate required for inhibition of 50% of the enzymatic activity of purified erythrocyte deaminase were 4.0 mM and 1.0 μM, respectively (data not shown). At levels of 5′-AMP and KCl which are in the physiological range (100 μM and 150 mM, respectively), the phytate concentration required for 50% inhibition of the activity of the purified enzyme was only 40 μM, approximately 100-fold less than the cellular levels of this modifier (Isaacks et al., 1976). While synergistic and antagonistic effects between phytate and other effectors (e.g., ADP, ATP, and GTP) have not been examined, it seems clear that phytate ion is a major allosteric negative modifier of avian erythrocyte AMP deaminase and that the enzyme in this cell type probably functions at a small fraction of its potential maximum activity. Under comparable experimental conditions, i.e., 100 μM 5′-AMP and 134 mM KCl, the concentration of phytate required to inhibit 50% of the activity of the muscle isozyme was approximately 1 μM, suggesting that this isozyme is much more sensitive than the erythrocyte deaminase to this effector. However, the presence of phytate in avian muscle (or any tissue other than erythrocytes) has not been demonstrated. Therefore, the possible physiological significance of phytate inhibition of the muscle-type AMP deaminase (whether this isozyme is found in muscle or other nonmuscle tissues; Sammons and Chilson, unpublished observations) cannot be assessed, at present.

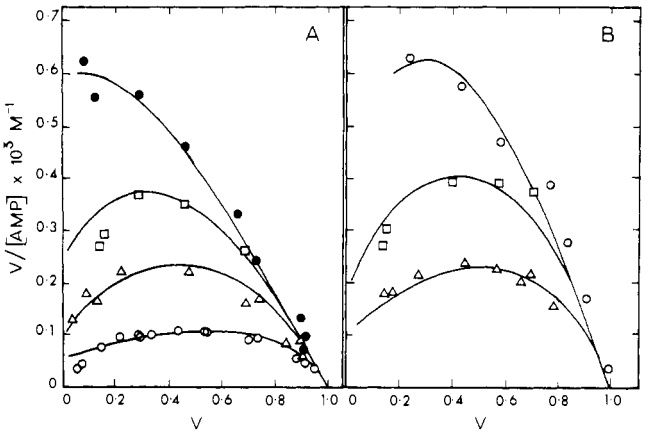

FIGURE 4: (A) The effect of potassium chloride on saturation with 5′-AMP. Enzymatic activity was determined by the discontinuous assay of ammonia production at 37 °C in 50 mM Tris–succinate (pH 6.5) as a function of substrate concentration in the absence and at three different levels of KCl. Experimental data are plotted for $v/[5'\text{-AMP}]$ vs. v. K_1 for 5′-AMP, determined from the slope at high substrate concentration in the presence of 10 mM KCl, is 1.8×10^{-3} M. Solid lines represent theoretical curves drawn according to eq 1, assuming values of n, c, and L as indicated at varying concentrations of KCl: (○) no KCl, $n = 4$, $c = 0.06$, $L = 355$; (△) 1 mM KCl, $n = 4$, $c = 0.06$, $L = 22$; (□) 3 mM KCl, $n = 4$, $c = 0.06$, $L = 3.6$; (●) 10 mM KCl, $n = 4$, $c = 0.06$, $L = 0.65$. (B) Inhibition of erythrocyte AMP deaminase by phytic acid. Enzymatic activity was determined as described in A, at two levels of phytic acid at a constant concentration of KCl (30 mM). K_1 for 5′-AMP, determined as described in A, for this preparation was 6.83×10^{-4} M. Solid lines represent theoretical curves drawn according to eq 1: (○) no phytic acid, $n = 4$, $c = 0.06$, and $L = 3.5$; (□) 5 μM phytic acid, $n = 4$, $c = 0.06$, and $L = 15$; (△) 20 μM phytic acid, $n = 4$, $c = 0.06$, and $L = 120$.

Yoshino et al. (1973) showed that avian erythrocyte AMP deaminase, like the muscle isozyme, is inactivated by reagents which are known to have a high affinity for Zn(II); we have made similar observations (data not shown). It has also been shown that dietary phytic acid can influence dietary requirements for this trace metal (Makdani et al., 1975; Morris and Ellis, 1976). These observations suggested that the sensitivity of erythrocyte AMP deaminase to phytic acid might be explained by a mechanism involving the formation of a Zn(II)–phytate complex. If this were the mechanism of inhibition, it would be expected that this inhibition would exhibit characteristics which are similar to those observed when enzyme is treated with orthophenanthroline or EDTA. Although reversible by Zn(II), inactivation via these reagents is time dependent, and once inactivated, enzymatic activity is not regenerated by dilution, high substrate concentration, or allosteric effectors. Among the characteristics of phytate inhibition observed during these studies were: (1) inhibition was instantaneous and was not enhanced after incubation with 15 mM phytic acid for as long as 30 min prior to dilution and assay at a low phytate concentration; (2) the same V_{max} was observed with or without phytic acid; and (3) inhibition was decreased by KCl. We cannot rule out the possibility that inhibition by

TABLE II: Amino Acid Composition of AMP Deaminase from Chicken Erythrocytes and Breast Muscle.

		erythrocyte preparations		muscle preparations	
amino acid	residue	I:[a] nearest integer (per 69 000g)	II:[b] nearest integer (per 69 000g)	III:[b] nearest integer (per 69 000g)	IV:[c] nearest integer (per 69 000g)
Trp	5.3[d]	5	5	4	6[d]
Lys	46.4	46	44	40	41
His	18.8	19	17	21	22
Arg	32.1	32	31	32	33
Asp/NH_2[e]	62.0	62	62	62	62
Thr[f]	27.4	27	30	27	27
Ser[f]	40.6	41	43	33	38
Glu	73.0	73	68	64	67
Pro	34.9	35	34	35	31
Gly	29.2	29	29	28	29
Ala	36.0	36	36	42	42
½-Cys[g]	9.9	10	nd	nd	8
Val	34.5	35	33	34	34
Met	16.3	16	16	18	16
Ile	25.4	25	23	23	24
Leu	61.9	62	63	57	54
Tyr[f]	27.3	27	32	32	33
Phe	24.8	25	23	29	30
total res		605	589 (599[h])	581 (589[i])	597
res wts		69 356	68 615[h]	67 757[i]	68 863

[a] HCl; average of duplicate analyses for hydrolysates of 24, 48, and 72 h, prepared by the method of Moore et al. (1958). [b] Mercaptoethanesulfonic acid; average of duplicate analyses for hydrolysates of 24, 48, and 72 h, prepared by the method of Penke et al. (1974). The analysis was performed on a different preparation than shown in *a*. Analysis of a single 24-h hydrolysate, prepared in 6 N HCl as by Moore et al. (1958) indicated similar relative proportions of amino acid residues (data not shown). [c] HCl; data taken from Boosman and Chilson (1976). [d] Determined by the method of Edelhoch (1967). [e] Set equal to 62.0. [f] Corrected for decomposition by extrapolation of values obtained from 24-, 48-, and 72-h hydrolysates, [g] Determined as cysteic acid after performic acid oxidation as described by Moore (1963). [h] Assuming ten residues of ½-Cys. [i] Assuming eight residues of ½-Cys.

this ligand involves binding to a zinc atom on the enzyme. However, it is clear that it does not involve removal of this essential cofactor (data not shown).

Amino Acid Composition. The amino acid compositions of two preparations of erythrocyte AMP deaminase are shown in Table II. Integral values for amino acids based on analyses of hydrolysates prepared in 6 N HCl and 3 N mercaptoethanesulfonic acid (preparations I and II, respectively) were in good agreement. Duplicate analyses of the 24-h 6 N HCl hydrolysates of preparation II gave integral values of amino acid residues very similar to those shown, except for glutamic acid, where 72 residues were found (data not shown). For comparison, the compositions of two preparations of AMP deaminase from adult breast muscle (isolated as referenced under Experimental Procedure) are also shown. Preparation III was obtained in this study and was hydrolyzed in 3 N mercaptoethanesulfonic acid. The composition of the preparation, designated as IV, taken from a previous publication from this laboratory (Boosman and Chilson, 1976), is in good agreement with that of preparation III; the small differences observed for serine and tryptophan are within experimental error for these residues. In each case examined (preparations II and III), hexosamine contents were found to be less than one residue per 69 000 (data not shown).

For most amino acids, the compositions of the erythrocyte and muscle AMP deaminases are very similar, but the red blood cell enzyme contains additional residues of half-cystine and leucine (two and six, respectively), and fewer residues (i.e., six each) of alanine and phenylalanine.

The erythrocyte and muscle AMP deaminases are also similar with respect to partial specific volume (0.730 and 0.735 cm^3/g, respectively) calculated from data in TABLE III and the ratio of polar to nonpolar residues (1.33 and 1.21, respectively). In each case, the sum of the residue weights yields subunit molecular weights based on amino acid composition that are in good agreement with the subunit size determined by $NaDodSO_4$-gel electrophoresis.

One of the more interesting questions regarding the molecular basis of AMP deaminase isozymes is the degree of sequence homology between these proteins. The possibility that the kinetic and immunological differences that have been described arise from posttranslational modification(s) cannot be rigorously ruled out by the data presently available. Harris and Teller (1973) have described an empirical relationship between amino acid composition and sequence homology for proteins that are functionally similar. It was suggested that when two proteins are similar, functionally, as well as by other criteria, the value of composition divergence (D) can be used to estimate the degree of sequence homology.

Although clearly speculative, it was of interest to compare the compositions of the preparations obtained in these studies with those published in the literature, on the basis of composition divergence, D. Different preparations of muscle AMP deaminase from the same or different sources, whether isolated and analyzed in this laboratory or by others (Coffee and Kofke, 1975; Wolfenden et al., 1968), exhibit D values ranging from 0.015 to 0.017. Similarly, D for the two preparations of erythrocyte AMP deaminase determined in this study is 0.016. The divergence factor for rat and rabbit skeletal muscle is 0.016; thus, it is apparent that these mammalian muscle AMP deaminases are very closely related, as was suggested by Coffee and Kofke (1975) on the basis of similarities in compositions (D not reported). The degree of sequence homology suggested by this value for composition divergence (i.e., 0.016) and Figure 4 of Harris and Teller (1973) is greater than 90%. A high degree of sequence homology between avian (chicken)

and mammalian (rat and rabbit) muscle AMP deaminases was also suggested from the composition divergences; however, the slightly higher D values of 0.022 and 0.026 suggest less homology than for rat and rabbit enzymes. The D values calculated from the amino acid compositions of the avian muscle and red blood cell AMP deaminases presented in Table II suggest a minimum of 85% sequence homology for these isozymes.

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