Adenylate Kinase of Human Erythrocyte

ISOLATION AND PROPERTIES OF THE PREDOMINANT INHERITED FORM*

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SUMMARY

Adenylate kinase exists in the human erythrocyte in a number of molecular forms with two alleles at a single polymorphic locus coding for most of the enzyme forms. The predominant enzyme form, AK a, was purified to constant specific activity in excess of 3000 and appeared homogeneous by chromatography, electrophoresis, and ultracentrifugation. Sedimentation velocity and partial specific volume measurements of AK a yielded values of \( s_{20, w} = 2.1 \) S and 0.722 cm\(^3\) per g. The molecular weight of the native enzyme was estimated to be 22,500 by sedimentation equilibrium and gel filtration analyses. The molecular weight of the denatured enzyme did not differ, indicating an absence of subunit structure in confirmation of genetic evidence of a single locus coding for the enzyme. The isolated enzyme demonstrated remarkable stability to denaturants (heat, guanidine HCl, urea) in the presence of appropriate stabilizing agents and could not be distinguished from rabbit muscle enzyme on this basis (as well as by a number of other kinetic and physicochemical parameters).

The erythrocyte adenylate kinases have a common molecular size but differ in their charge properties. They demonstrate anomalous electrophoretic behavior, migrating anionic to hemoglobin in starch gel, yet exhibit isoelectric points considerably alkaline to hemoglobin (e.g. AK a, \( pI = 9.0 \)) by isoelectric focusing.

Adenylate kinase (ATP-AMP phosphotransferase, EC 2.7.4.3), an apparently ubiquitous enzyme, is present at high activity levels in many cells, including the human erythrocyte. The enzyme catalyzes an equilibrium of the adenine nucleotides, providing a unique buffering role against rapid concentration changes of any one component of this pool. The enzyme also serves as a primary regulating agent of these reactions involving participation of adenine nucleotides as substrate, activator, or inhibitor. Adenylate kinases have been prepared in either crystalline or apparent homogeneous form from rabbit (1) and squid (2) muscles, yeast (3), and bovine (4) and rat (5) liver.

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Adenylate kinase exists in a number of molecular forms in human tissues (6). Genetic studies indicate that two alleles at a single polymorphic locus code for most of the different forms of the enzyme in human erythrocytes (7), with two common phenotypes, AK 1 (homozygote) and AK 2-1 (heterozygote) exhibited at frequencies of around 90 and 10% within a large United States population (8). The most common phenotype, AK 1, exhibits a major and several minor electrophoretically distinguishable enzyme forms. The less common phenotype, AK 2-1, exhibits two major and again several minor associated enzyme forms.

The indicated presence of multiple molecular forms of adenylate kinase in human erythrocyte prompted a study directed to their separation and characterization. Purification to apparent homogeneity of the primary enzyme component of the most common phenotype, AK 1, is described in the present report. The isolated enzyme demonstrated an absence of subunit structure consistent with genetic evidence (7, 8) for the involvement of a single locus in its production. Contrary to an apparent near neutral isoelectric point, indicated by electrophoretic patterns exhibited in starch gel (7-9), the isolated enzyme showed strongly basic properties with ion exchange celluloses and a \( pI \) of 9.0 by isoelectric focusing technique. Additional specific properties of the enzyme are detailed in this report.

EXPERIMENTAL PROCEDURES

Materials—Outdated human blood from the Peninsula Blood Bank, Burlingame, Calif., served as the source of erythrocyte adenylate kinase. Whatman CM-cellulose and DEAE-cellulose in microgranular form were from Reeve Angel. Glucose-6-P dehydrogenase type XI, hexokinase type C-300, pyruvate kinase type II, lactate dehydrogenase type XI, hexokinase type C-300, pyruvate kinase type II, lactate dehydrogenase type XI, Triton X-100, and Tween 80 were from Sigma. Ultrapure urea, Tris, sucrose and trilithium ADP were from Schwarz-Mann.

Enzyme Measurements—Adenylate kinase activity was measured as the rate of ATP formation determined in the forward direction 2 ADP \( \rightarrow \) ATP + AMP. ATP formation was determined fluorimetrically by enzyme-coupled NADP reduction (10, 11). Reaction mixtures contained 50 mM Tris buffer, pH 8.0, 1 mM ADP, 2 mM MgCl\(_2\), 0.1 mM NADP, 2 mM glucose, 0.1 unit of hexokinase, and 0.1 unit of glucose-6-P dehydrogenase, all in a 1-ml reaction volume. Reactions were carried out at 25° using a model III Turner fluorometer equipped with a temperature-stabilized sample compartment and an attached Beckman linear recorder. Maximal preservation of enzyme activity required dilution for assay in medium containing surface active agent (0.2% Triton X-100), EDTA (0.1 mM), dithiothreitol (0.5 mM), and excess electrolyte (0.1 M KCl plus 10 mM NaCl, buffer at pH 7). One unit of adenylate kinase activity is defined as the amount of enzyme...
catalyzing the formation of 1 mol of ATP per min at 25°C under the specified conditions of assay. Adenylate kinase activity also was determined by the back reaction in which ADP formation was measured fluorimetrically by enzyme-coupled oxidation of NADH. Reaction mixtures contained 50 mM Tris, pH 8.0, substrates (ATP, AMP) and MgCl₂ at specified concentrations, 0.1 mM phosphoenolpyruvate, 0.5 mM NADH, 50 mM KCl, 1 unit of pyruvate kinase, and 1 unit of lactate dehydrogenase in a 1.0 ml reaction volume.

Protein Measurement—Hemoglobin was estimated as the cyano-methemoglobin complex. Protein was routinely estimated by absorbance at 280 nm at neutral pH or at 250 nm in 0.1 N NaOH (to avoid interference by AMP when present), assuming in each case an molar absorbance coefficient, ε₂₈₀ = 80. In the case of the homogeneous enzyme an absorbance coefficient, ε₂₈₀ = 5.6, was established based on a refractometric method with the use of Raleigh interference optics as a primary measure of enzyme protein concentration. Relative protein concentration also was determined at higher sensitivity by fluorescence measurements employing an Amino-Beckman spectrophotofluorometer with excitation at 287 nm and emission measured at 325 nm. Absorption spectra of the enzyme were measured employing a Beckman model DB spectrophotometer with recorder attached.

Gel Filtration and Column Chromatography—A preparative column of Sephadex G-100 and analytical columns of Sephadex G-200 and G-75 were employed. Columns were equilibrated and developed at near 0°C by reverse flow employing a peristaltic pump (Perpex pump LKB) at set flow rates. DEAE-cellulose was equilibrated in Tris-chloride at pH 8.5 and CM-cellulose in sodium phosphate at pH 7.0. Following equilibration, excess ions were removed and the DEAE-cellulose was resuspended in final 2 mM Tris-chloride at pH 8.5 and the CM-cellulose in final 2 mM sodium phosphate at pH 7.0. The suspensions were prepared to contain 0.5 packed volume of exchange on light centrifugation.

Isoelectric Point—Isoelectric focusing was carried out in an LKB 8101 electrofocusing column of 110 ml volume at near 0°C. A sucrose density gradient containing 1% carrier ampholytes, 0.01% Tween 80, and 0.1% diisobutanol was layered between the electrode solutions.

Acrylamide Gel Electrophoresis—Disc electrophoresis was carried out at near 0°C as described by Ornstein and Davis (14) employing a 7.5% resolving gel and sample application in 10% sucrose solution. Electrophoresis in sodium dodecyl sulfate (SDS) was carried out by a modification (15) of the system described by Shapiro et al. (16). Gels were prepared to contain 10% acrylamide, 0.1% SDS, 0.1% mercaptoethanol, and 0.1% Tris, pH 7.3, and 8 M urea. The buffer mixture contained 0.1% SDS, 0.1% mercaptoethanol, and 0.1% Tris, pH 7.3. Samples contained 10 to 20 μg of protein in 20 μl volume of 0.1 M Tris, pH 7.3, 8 M urea, 1% SDS, and 0.1% mercaptoethanol. Electrophoresis was carried out for 8 hours at 70°C per gel. All gels were fixed in 10% trichloroacetic acid and stained with Coomasie blue.

Starch Gel Electrophoresis—Electrophoresis was carried out in starch gel at pH 7 using the buffer system and specific staining employing a 7.5% resolving gel and sample application in 10% sucrose solution. Electrophoresis in sodium dodecyl sulfate was developed at near 0°C by reverse flow employing a peristaltic pump (Perpex pump LKB) at set flow rates. DEAE-cellulose was developed at near 0°C by reverse flow employing a peristaltic pump (Perpex pump LKB) at set flow rates. DEAE-cellulose was equilibrated in Tris-chloride at pH 8.5 and CM-cellulose in final 2 mM Tris-chloride at pH 8.5 and the CM-cellulose in 8 M urea. The suspensions were prepared to contain 0.5 packed volume of exchange on light centrifugation.

Ultracentrifuge Studies—Measurements were made in the Beckman-Spinco model F analytical ultracentrifuge equipped with electronic speed control and photoelectric scanning accessory and multiplexer. Sedimentation velocity experiments were carried out at a rotor speed of 60,000 rpm. Standard sedimentation coefficients, s₂₀, were computed in the usual fashion. Boundary positions were taken to be the maximum ordinate of the peak for purposes of enzyme stabilization. Electrophoresis was carried out at near 0°C.

The molecular weight of the enzyme in denaturing concentrations of guanidine hydrochloride (5 M) was determined from sedimentation equilibrium experiments. Schlieren optics were used and results were evaluated by application of the method of Low and Stadler (17) to obtain a value of 0.71 cm² per g for the enzyme in the denaturing reagent.

### RESULTS

Isoelectric Point of Erythrocyte Adenylate Kinase a (AK a)

Procedures employed for the isolation of AK a, the predominant molecular form of the human erythrocyte enzyme, are detailed in the following sections. A stepwise summary of the purification protocol leading to the isolation of the enzyme from a 2-liter batch of erythrocytes is presented in Table 1.

**Preliminary Typing—**Outdated human blood samples were preliminarily screened by starch gel electrophoresis to separate approximately 1 out of 10 blood samples exhibiting the less common AK 2-1 phenotype (7-9). Electrophoretic patterns distinguishing the less common AK 2-1 phenotype (initial sample, left to right) from among nine randomly selected blood samples are shown in Fig. 1. The predominant, AK 1 phenotype, displays a major (designated AK a) and associated minor (AK b, AK c) enzyme forms. Blood samples exhibiting the AK 1 phenotype were combined and the erythrocytes were separated and employed directly, or after storage in the frozen state, for enzyme preparation.

Chloroform-Methanol Denaturation—A 2 liters of washed erythrocytes was added an equal volume of water and the resulting hemolysate was cooled to 2-4°C. To the hemolysate were added concentrated ammonium hydroxide solution to pH 8.7, AMP to 1 mol (for enzyme stabilization), and 1200 ml of an equal mixture (v/v) of chloroform-methanol, previously cooled to -20°C. The mixture was immersed in an ice-water bath and

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tbody>
<tr>
<td><strong>Purification Protocol of Erythrocyte Adenylate Kinase a</strong></td>
</tr>
<tr>
<td>Protein was estimated by hemoglobin measurement through Steps a to c, by optical density at 290 nm in 0.1 N KOH through Steps d to f, and by optical density at 280 nm at pH 7.0 through Steps g to i.</td>
</tr>
<tr>
<td>Fraction</td>
</tr>
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<td>---</td>
</tr>
<tr>
<td>a. Hemolysate, 1:1</td>
</tr>
<tr>
<td>b. Chloroform-methanol concentrate (1)</td>
</tr>
<tr>
<td>c. Chloroform-methanol concentrate (2)</td>
</tr>
<tr>
<td>d. Sephadex G-100 dialyzed concentrate</td>
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<tr>
<td>e. DEAE-cellulose filtrate.</td>
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<tr>
<td>f. CM-cellulose batch eluate</td>
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<tr>
<td>g. CM-cellulose chromatography (1)</td>
</tr>
<tr>
<td>h. CM-cellulose chromatography (2)</td>
</tr>
<tr>
<td>i. CM-cellulose chromatography (3)</td>
</tr>
</tbody>
</table>

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
stirred at 5-min intervals over a 1-hour period. Following the addition of 2.8 liters of cold water (near 0°), the mixture was centrifuged to remove denatured hemoglobin. To the resulting red solution were added 1 N acetic acid to pH 7, Triton X-100 to 0.002%, and caprylic acid to 0.01% (antifoaming agent). The solution was concentrated under reduced pressure, using a rotary evaporator, initially at room temperature until organic solvents had boiled off, then at elevated temperatures of 30-35°. Globin per ml of solution.

**Fractionation on Sephadex G-100** — The concentrated enzyme solution was centrifuged to remove denatured protein and divided into two equal aliquots. Each aliquot (35 ml) was fractionated separately on a preparative column of Sephadex G-100 in a medium containing 50 mM KCl, 20 mM P; buffer (pH 7.0), 0.1 mM EDTA, and 0.002% Triton X-100. The active enzyme fractions from both column runs were pooled and concentrated under reduced pressure in a rotary evaporator at 30° to about 40 ml volume. The concentrated enzyme solution was dialyzed overnight at near 0° with stirring against 2 liters of a solution containing 10 mM P; buffer at pH 7.0, 0.1 mM EDTA, 0.002% Triton X-100, and 0.1 mM dithiothreitol (Table I, Step d).

**Treatment with DEAE-cellulose and Batch Absorption and Elution from CM-cellulose** — To the dialyzed enzyme solution were added 9 volumes of a cold solution of 0.1 mM EDTA plus 0.002% Triton X-100 and immediately 175 ml of a DEAE-cellulose suspension (1 ml/40 mg of protein). Prior to use, the DEAE-cellulose was equilibrated at pH 8.5 in Tris-chloride buffer and suspended to contain 0.5 packed volume of exchanger on light centrifugation in final 2 ml of the buffer. The resulting mixture was filtered quickly through a 500-ml capacity, coarse, scintillated glass funnel. The filtrate was collected directly into a pressure flask containing 175 ml of a CM-cellulose suspension in 2 mM P; buffer at pH 7.0, 0.1 mM EDTA, and 0.002% Triton X-100, in order to affect immediate adsorption of enzyme. The CM-cellulose was prepared for use by equilibrating with P; buffer at pH 7.0 and was resuspended to contain 0.5 packed volume of the exchanger on light centrifugation. Adsorption of adenyate kinase on CM-cellulose remained incomplete with a minor fraction of the activity (around 10%) left unadsorbed. Examination of this unadsorbed enzyme activity by starch gel electrophoresis revealed that it consisted primarily of AK b (see Fig. 1): A small aliquot of the enzyme solution on mixing with DEAE-cellulose was filtered separately and the filtrate was diluted immediately in enzyme stabilizing medium (see “Experimental Procedures”) and assayed for enzyme activity and protein content (see Step e, Table I). Due to a pronounced instability of the enzyme in media of low ionic strength at this stage of the purification, it was necessary that all operations up to the adsorption of enzyme on CM-cellulose be accomplished quickly and at temperatures maintained at near 0°.

The CM-cellulose, containing adsorbed enzyme, was collected in a 125-ml volume scintillated glass funnel of coarse porosity and washed with several volumes of a medium containing 2 mM P; pH 7.0, 0.1 mM EDTA, and 0.002% Triton X-100, with care taken to obtain an even spread of the exchanger in the funnel. Enzyme elution was carried out by passing eluting medium (0.1 mM P; at pH 7.0 containing 0.1 mM EDTA, 0.2 mM dithiothreitol, and 0.002% Triton X-100) through the exchanger by gravity flow. The enzyme was collected within the initial 75-ml elution volume. An approximate 40-fold purification of the enzyme was achieved by a combination of the batch DEAE-cellulose and CM-cellulose steps (see Steps e and f, Table I).

**First Chromatography on CM-Cellulose** — Enzyme solution obtained by batch elution from CM-cellulose was concentrated by ultrafiltration with a UM-10 Amicon filter to less than 10-ml volume, transferred to cellophane tubing of 1.0-cm diameter, and dialyzed for 3 hours with stirring against 1 liter of solution containing 10 mM Tris buffer, pH 7.5, 0.002% Triton X-100, 0.5 mM dithiothreitol, and 0.2 mM EDTA. The resulting dialyzed enzyme solution was diluted with an equal volume of water and immediately transferred to a CM-cellulose column for fractionation. All procedures prior to and accompanying the fractionation were carried out at near 0° to minimize enzyme inactivation. Enzyme elution was accomplished by applying a linear gradient of increasing Tris concentration and fractions were collected into tubes containing pre-added salt to increase ionic strength to provide for enzyme stabilization. Enzyme was found to be eluted within an initial minor activity peak followed by a second major peak of activity, designated as AK b and AK a, respectively, (Fig. 2A). Fractions comprising AK b were pooled and utilized without further purification in studies presented in later sections of this report. Fractions comprising the major enzyme peak AK a were combined and, although demonstrating an average specific activity in excess of 1000-fold, were still ob-

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**Fig. 1. Enzyme profiles of erythrocyte adenylate kinases, exhibited by nine randomly selected samples of human blood, following separation by electrophoresis in starch gel. Electrophoretically separated enzyme forms are designated a, b, c, and d. The single blood sample to the extreme left, contains an additional variant enzyme, Form d. The remaining blood samples contain the common enzyme forms with a representing the major and b and c the quantitatively lesser forms.**
viously impure. The combined fractions were concentrated by vacuum filtration to about 2-ml volume and dialyzed (1-cm diameter cellophane tubing) for 3 hours with stirring against 10 mM Tris, pH 7.5, 0.1 mM EDTA, 0.002% Triton X-100, and 0.25 mM dithiothreitol. Fractions of 2.0-ml volume were collected at a constant elution rate of 12 ml per hour into prepared tubes containing 0.2 ml of 0.5 mM phosphate buffer at pH 7.5 to effect enzyme stabilization by raising ionic strength.

Second Chromatography on CM-Cellulose—The dialyzed enzyme solution was diluted with an equal volume of water and transferred immediately to a CM-cellulose column. Enzyme elution again was carried out by a linear gradient of increasing Tris concentration at pH 7.5. Enzyme activity was eluted within a narrow, almost coincident band of protein, suggesting a probable high degree of enzyme purity (Fig. 2B). The active enzyme fractions were combined and reduced to approximately 1-ml of volume by vacuum filtration using 1-cm diameter cellophane tubing. The concentrated enzyme solution was dialyzed for 3 hours with stirring against 1 liter of 0.5 mM Tris, pH 7.5, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.002% Triton X-100.

Resolution to Constant Specific Activity on CM-Cellulose—The dialyzed enzyme solution was diluted with an equal volume of water and chromatographed for a third time on a column of CM-cellulose. The chromatography resulted in only little further increase in enzyme specific activity, yielding a single, apparently homogeneous enzyme-protein peak as shown in Fig. 3. The specific activity of the combined enzyme fractions exceeded 3000 pmol of substrate conversion per min per mg of protein, determined at 25° by the standard assay employed (see "Experimental Procedures"). The combined fractions were concentrated by vacuum filtration to 1.0 ml and represented the final isolated product (see Table I, Fraction i).

The isolated enzyme product was combined with other comparable enzyme preparations, yielding a total of approximately 20 mg of enzyme protein, and stored in about 0.6 saturated ammonium sulfate at -20°, following unsuccessful attempts to obtain enzyme crystallization in this medium. Overall yield among a number of enzyme preparations varied between 10 and 20%, with the greater portion of these losses resulting due to enzyme instability accompanying fractionation procedures requiring media of low ionic strength.

### Enzyme Purity

A high degree of enzyme purity was assumed since specific activity values of the products of the final preparative steps appeared to approach constancy. Furthermore, the specific activity of the final enzyme product exceeded by severalfold or equaled that of apparent, homogeneous adenylate kinases prepared from various other sources. Erythrocyte AK a was subjected to rigorous analyses for purity and appeared homogeneous by each of the criteria detailed below.

#### Homogeneity Analysis by Chromatography on Carboxymethyl-cellulose—Repeate Fractionation of AK a on CM-cellulose resulted in removal of obvious residual contaminants, yielding an apparent homogeneous product. Evidence of enzyme homogeneity was obtained by specific activity measurements of successively eluted enzyme fractions (see Fig. 3). Specific activity was found to remain essentially constant across the enzyme peak and was maintained at values of around 3000.

#### Homogeneity Analysis by Acrylamide Gel Electrophoresis—Purification sequences of AK a were followed by electrophoretic analysis of the successively prepared enzyme fractions. The presence of AK a was not evident in the protein patterns prior to the final purification steps, involving column chromatography on CM-cellulose, as illustrated by the stained gels shown in Fig. 4. The final chromatographically homogeneous, enzyme product appeared as a single, diffuse protein band (Gel 6). Attempts to sharpen the enzyme band by carrying out the electrophoresis in the presence of ATP, ADP, AMP, magnesium, mercaptoethanol, and EDTA either singly or in various combinations were not successful. On the other hand, prior denaturation of enzyme in urea and SDS and electrophoresis in the presence of these agents resulted in a single, narrow migrating band of protein (see below), indicative of product homogeneity.

#### Homogeneity Analysis by Gel Filtration—An aliquot of AK a of specific activity 3000 units per mg resulting from fractionation...
FIG. 4. Polyacrylamide gel disc electrophoretic patterns of successively purified fractions of adenylate kinase. Fractions: 1, hemolysate; 2, product of second chloroform-methanol de- naturation; 3, product of Sephadex G-100 fractionation; 4, CM- cellulose filtrate; 5, CM-cellulose eluate; and 6, final chromatographically ‘homogeneous enzyme product. All fractions contained approximately 50 µg of protein except the final product which contained 15 µg of enzyme.

FIG. 5. Homogeneity analysis of adenylate kinase a on Sephade x G-100. Adenylate kinase a of specific activity around 3000 was applied in a 2.0-ml volume to a column (2.5 X 100 cm) of Sephadex G-100. The elution rate was maintained at 35 ml per hour and 3.5-ml fractions were collected. The elution medium contained 0.075 M NaCl, 10 mM Pi at pH 7.0, 0.1 mM EDTA, and 0.01% Tween 80. The specific activity of successively eluted enzyme fractions was determined. Protein was measured by its native fluorescence (see “Experimental Procedures”).

on CM-cellulose (see Fig. 3) was analyzed further by gel filtration. Fractionation on a column of Sephadex G-100 yielded a single coincident peak of enzyme activity and protein concentration with successively eluted fractions of constant specific activity resulting (see Fig. 5).

Homogeneity by Sedimentation—Sedimentation studies of AK a revealed a single symmetrical peak in sedimentation velocity studies without evidence of heterogeneity. Additional evidence of homogeneity was obtained in sedimentation equilibration experiments in which plots of ln c versus t² were found to be linear (see below).

FIG. 6. Thermal stability properties of adenylate kinase a. A, enzyme solutions containing 0.1 unit of enzyme were heated for 10 min at 90° in capped tubes in the presence of 10 mM Pi buffer, pH 7.0, KCl concentrations as indicated, and additional agents as follows: Curve 1, 0.2 mM EDTA, 0.02% Triton X-100, and 2 mM dithiothreitol; Curve 2, 0.02% Triton X-100, and 2 mM dithiothreitol; Curve 3, 0.2 mM EDTA, 2 mM dithiothreitol; and Curve 4, 0.2 mM EDTA and 0.02% Triton X-100. Residual enzyme activity was determined. B, enzyme solutions containing 0.1 unit of en zyme were heated for 10 min at 90° in capped tubes containing 0.5 M KCl, 0.2 mM EDTA, 0.02% Triton X-100, 2 mM dithiothreitol, and buffer solutions at 20 mM concentration. Buffers were acetate (pH 4, 5) phosphate (pH 6, 7), and Tris (pH 8, 9, 9.5). Residual enzyme activity was determined.

Enzyme Properties

Stability Characteristics—Enzyme preparations purified to homogeneity maintained maximal specific activities at around 3000 for only a few days, dropping soon thereafter to around 1000 during storage at near 0°. Specific activity was maintained at this lower level for prolonged storage periods at -20° in 0.6 saturated ammonium sulfate solution.

Enzyme instability was encountered in dilute solutions of even the crude hemolysate. Enzyme was, therefore, routinely diluted for assay in medium containing added protective agents which included Triton X-100, EDTA, dithiothreitol, and electrolyte. Enzyme instability was extended with progressive purity from the dilute to the more concentrated solutions and was particularly pronounced in media of low ionic strength. On the other hand, in the presence of added protective agents, solutions of the purified enzyme demonstrated remarkable stability on exposure to extremes of both temperatures and pH as illustrated in Fig. 6, A and B. Only minimal losses of enzyme activity were recorded even at boiling water temperatures. Furthermore, in the presence of the designated protective agents, the enzyme showed little additional inactivation on heating at 90° in 5 M guanidine hydrochloride (not shown). The enzyme was found to be considerably less stable to heat in the impure state with higher concentrations of dithiothreitol required for protection.

Electrophoretic Behavior—Electrophoresis at pH 7.0 in starch gel of crude hemolysate (AK 1 phenotype) resolves a major enzyme component remaining near the origin and two (or more) minor components migrating as anions, in contrast to cationic migration of hemoglobin (see Fig. 1). Chromatography on CM-cellulose resolves the major and a minor adenylate kinase component (designated AK a and b, see Fig. 2 A), which are identified relative to their counterparts in crude hemolysate (see Fig. 1) by comparative electrophoretic migration in starch gel as shown in Fig. 7. Resolution of AK a and b by cation exchange chromatography on CM-cellulose at pH 7.5 indicated not only their dif-
Fig. 7. Comparative electrophoretic mobility in starch gel of isolated forms a and b of adenylate kinase and their counterparts in the original crude hemolysate. Isolated enzyme forms a and b as well as crude hemolysate were diluted to contain 5 units of activity per ml in 10% serum (serum added to stabilize enzyme) and applied to the gel on filter paper strips. Electrophoresis was carried out at pH 7.0 for 5 hours at 40-ma constant current. Enzyme stain was developed for about 1 hour prior to photography.

ferences in net charge, but also their cationic nature at this pH (Fig. 2A), contrary to their neutral to anionic electrophoretic behavior in starch gel at pH 7. (see Figs. 1 and 7). In view of these contradictory charge properties exhibited by erythrocyte AK a and b, their isoelectric points were examined by isoelectric focusing technique. In Fig. 8A is shown the isoelectric pattern of a hemolysate sample (AK 1 phenotype) in which a major and two minor enzyme forms have been resolved cathodal to hemoglobin with indicated isoelectric points of 9.0, 8.7, and 8.5, respectively. In Fig. 8B are shown superimposed patterns of separate isoelectric focusing runs carried out on purified preparations of AK a and AK b. Respective isoelectric points of 9.0 and 8.7 are exhibited and identification of each enzyme form in relation to its counterpart in the crude hemolysate is obtained based on commonality of isoelectric points.

The isoelectric focusing experiments thus serve to confirm the highly basic nature of the erythrocyte adenylate kinases, contrary to their observed electrophoretic behavior in the starch gel system.

Disc electrophoresis of AK a resulted in a diffuse migrating protein band (Fig. 4, Gel 6), suggesting the possible presence of monomer and dimer enzyme forms in rapid equilibrium during the electrophoresis. On the other hand, little evidence of enzyme aggregation was observed in the course of sedimentation or gel filtration analyses (see below).

Sedimentation Characteristics—Sedimentation coefficients of AK a were determined over a 30-fold concentration range, as shown in Table II. Over this range there was no apparent change in sedimentation rate with concentration, indicating a probable compact globular structure for the enzyme. An average value for $s_{20,w}$ of 2.1 S was obtained.

The results of simultaneous sedimentation equilibrium experiments in water and deuterium oxide are also summarized in Table II. Typical plots of $\ln c$ versus $r^2$ are shown in Fig. 9. From the average values obtained for the partial specific volume and effective molecular weights, a value of 21,000 was calculated for the molecular weight of native adenylate kinase. Since very

![Figure 8](http://www.jbc.org/)

**Table II**

Summary of sedimentation data and molecular weight determinations

<table>
<thead>
<tr>
<th>Determination</th>
<th>Observed value</th>
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<tbody>
<tr>
<td>Sedimentation velocity ($s_{20,w}$), schlieren at:</td>
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</tr>
<tr>
<td>6.0 mg/ml</td>
<td>2.1 S</td>
</tr>
<tr>
<td>1.2 mg/ml</td>
<td>2.1 S</td>
</tr>
<tr>
<td>Sedimentation velocity ($s_{20,w}$), scanner at:</td>
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<tr>
<td>1.3 mg/ml</td>
<td>2.0 S</td>
</tr>
<tr>
<td>1.2 mg/ml</td>
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</tr>
<tr>
<td>0.6 mg/ml</td>
<td>2.0 S</td>
</tr>
<tr>
<td>0.2 mg/ml</td>
<td>2.1 S</td>
</tr>
<tr>
<td>Partial specific volume, $\bar{e}$, native enzyme (3)$^a$</td>
<td>0.722 ± 0.004 cm$^3$/g</td>
</tr>
<tr>
<td>Molecular weight</td>
<td></td>
</tr>
<tr>
<td>Equilibrium sedimentation</td>
<td></td>
</tr>
<tr>
<td>Native enzyme (4)$^a$</td>
<td>21,300 ± 700</td>
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<tr>
<td>Enzyme in 5 M guanidine HCl (5)$^a$</td>
<td>23,400 ± 1700</td>
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<tr>
<td>Sephadex chromatography, native enzyme (4)$^a$</td>
<td>22,500 ± 1000</td>
</tr>
<tr>
<td>SDS-polyacrylamide elect (2)$^a$</td>
<td>23,000 ± 1000</td>
</tr>
</tbody>
</table>

$^a$ Numbers in parentheses refer to number of determinations used to calculate average values and standard deviations.
low concentrations of the enzyme were used in these studies and since little concentration dependence was indicated from the sedimentation velocity results, it can be concluded that this value is equivalent to the infinite dilution value.

For the enzyme in denaturing concentrations of guanidine hydrochloride, sedimentation equilibrium plots of ln r versus r^2 were typically curved upward, indicating that some aggregation of the enzyme occurs in this medium. Because of fractionation in the centrifuge cell, however, the data plots for five runs were linear over the upper third to half of the fluid column. Molecular weights calculated from these linear regions gave an average value of 23,400 (see Table II). There was no indication of dissociation of the native enzyme in guanidine hydrochloride.

**Gel Filtration Characteristics**—Fractionation of crude hemolysate on a column of Sephadex G-100 results in the elution of a single, symmetrical peak of adenylate kinase activity, consisting of all electrophoretically separable enzyme forms. Elution volumes of AK a and b determined separately on a calibrated column of Sephadex G-100 (19) were found to be indistinguishable, indicative of their common molecular size. The molecular weight of erythrocyte adenylate kinase determined by gel filtration technique with Sephadex G-75 and G-100 yielded an average size estimate of 22,500.

Electrophoresis in SDS-Polyacrylamide Gel—Electrophoresis of the enzyme-SDS complex in the presence of urea and mercaptoethanol yielded only one component, migrating as a single, symmetrical peak (Fig. 10A) of approximately 150,000 molecular weight. Immunological Properties—Repeated injections of erythrocyte AK a (600-µg aliquots in complete Freund’s adjuvant) into rabbit footpads at 3-week intervals failed to produce detectable antibody titer in two rabbits tested over a period of 3 months. The apparent lack of antigenicity of the human erythrocyte enzyme suggested the possibility of a high degree of structural similarity between human and rabbit enzymes.

**Table III**

<table>
<thead>
<tr>
<th>Substrate pair</th>
<th>Relative rate of ADP formation</th>
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<tbody>
<tr>
<td>AMP + ATP</td>
<td>100</td>
</tr>
<tr>
<td>AMP + dATP</td>
<td>52</td>
</tr>
<tr>
<td>AMP + CTP</td>
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</tr>
<tr>
<td>dAMP + ATP</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>CMP + ATP</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Analyzes of the amino acid compositions, electrophoretic properties, stability characteristics, and kinetic properties indicate a remarkable similarity in support of such a conclusion.

Catalytic Properties—Erythrocyte AK a and b demonstrated identical substrate specificity patterns as shown in Table III. In addition to adenine nucleotide, catalytic activity also was observed in the presence of deoxyadenosine and cytidine nucleotides to the extent indicated. No measurable catalytic activity resulted in the presence of AMP plus dGTP, GTP, UTP, ITP; or, ATP plus dGMP, GMP, UMP, IMP.

AK a and b could not be distinguished on the basis of various measured kinetic parameters, including Michaelis constants (K_m) for substrates AMP, ATP, and ADP. Kinetic analyses of the enzyme catalyzed bimolecular, reversible reaction included initial rate measurements as a function of substrate concentration. In Fig. 11, A and B, are shown double reciprocal plots of reaction velocity versus variable ATP and constant AMP concentrations and variable AMP and constant ATP concentrations, respectively. The plots resulted in each case in a series of straight lines intersecting at a common point above the x axis. Secondary plots of the data (see inset) allow evaluation of V_max and Michaelis constants (20). K_m values from these data are 9 and 8 x 10^-5 M for ATP and AMP, respectively. Magnesium concentration was maintained at a 2:1 ratio relative to the ATP concentration in these experiments. Repeating the experiments at constant 1.0 mM Mg^2+ concentration yielded similar linear
Fig. 11. A, relationship between initial velocity and variable ATP, constant AMP concentrations. Reaction mixtures contained 0.1 mM Tris (pH 8.0), ATP as designated, Mg$^{2+}$ at twice ATP concentrations, and AMP at (a) 0.2 mM, (b) 0.5 mM, and (c) 2.0 mM. Inset shows a secondary plot of the data with intersections of y and x axes yielding 1/V$_{max}$ and $-1/K_{ATP}$ values, respectively. The initial velocity is in nanomoles of ADP formed per min at 25°. B, relationship between initial velocity and variable AMP, constant ATP concentrations. Reaction mixtures contained 0.1 mM Tris, (pH 8.0), AMP as designated, Mg$^{2+}$ at twice ATP concentrations and ATP at (a) 0.25 mM, (b) 0.575 mM, (c) 0.5 mM, and (d) 0.75 mM. (Inset as in Fig. 12A.)

Fig. 12. A, Effect of magnesium ion concentration on reaction velocity. Reaction mixtures contained 0.1 mM Tris, pH 8.0, ADP at designated concentrations, and Mg$^{2+}$ to ADP ratios at 1, 0.5; 2, 1.0, and 3, 12, and Mg$^{2+}$ concentrations at 4, 1.0 mM and 6, 2.5 mM. The initial velocity is in nanomoles of ADP formed per min at 25°. Extrapolation of Curve 4 to the negative abscissa gives an estimated $K_{Mg}$ value of 1.1 x 10$^{-4}$ M with all other curves yielding corresponding larger values. B, inhibition of ADP conversion by the product AMP. Reaction mixtures contained 0.1 mM Tris, pH 8.0, ADP at designated concentrations, Mg$^{2+}$ at 1.0 mM, and AMP at 1, 0.2 mM; 2, 0.1 mM, and 3, 0.05 mM. The initial velocity is in nanomoles of ATP formed per min at 25°.

Plots and 10 to 20% greater apparent $K_m$ values for ATP and AMP. Further elevation of Mg$^{2+}$ concentration resulted in obvious inhibition of the reaction velocity, with increasing deviation from linearity observed in the reciprocal plots.

Selected initial rate measurements carried out in the direction of ATP and AMP formation with ADP as the variable substrate are shown in Fig. 12. The effect of increasing Mg$^{2+}$ concentration, maintained either as a ratio of the ADP concentration, or at constant levels, on the initial velocity is illustrated in Fig. 12A. A plot of reciprocal velocity versus the square of the reciprocal substrate concentration resulted in a series of straight lines of common intercept on the y axis (therefore common $V_{max}$). Maximum velocities were approached at Mg$^{2+}$ concentrations of around 1.0 mM, over the substrate range selected. Mg$^{2+}$ concentrations exceeding this level resulted in progressively increasing inhibition of the reaction velocity. The $K_m$ value for ADP in the presence of 1.0 mM Mg$^{2+}$ concentration was determined to be 1.1 x 10$^{-4}$ M. All other combinations of Mg$^{2+}$ to ADP concentrations tested resulted in straight lines of greater slope and therefore correspondingly larger apparent $K_m$ values. Inhibition by the product AMP on the initial velocity of ADP conversion is shown in Fig. 12B. Inhibition was found to be noncompetitive in nature when tested in the presence of 1.0 mM Mg$^{2+}$ concentration. In this regard, competitive inhibition of ADP conversion by AMP (as well as ATP) was observed with rabbit muscle enzyme (21) using Mg$^{2+}$ concentrations at one-half ADP concentrations.

**DISCUSSION**

Adenylate kinase exists in the human erythrocyte in multiple molecular form and comprises about 0.005% of the cell protein. Although most of the enzyme is associated with soluble protein, significant activity is retained with the stromal fraction as prepared in media of low ionic strength (e.g. see Ref. 22). The bound enzyme can be released from stroma by increasing ionic strength (0.8 M KCl) and does not differ from the major, soluble enzyme form. Genetic studies indicate involvement of a single polymorphic locus in the coding of erythrocyte adenylate kinase (7) with distribution of two common alleles among the population. The most common phenotype (AK 1) exhibits a single major (AK a) and several minor (AK b, AK c) association enzyme forms which can be distinguished by differences in their charge but not size. That the minor enzyme forms represent secondary products is indicated by the formation of similar derived products from the isolated AK a accompanying storage. An inability to demonstrate enzyme subunit structure (Fig. 10) confirms genetic evidence for a single locus coding for adenylate kinase (based on absence of hybrid enzyme forms in heterozygotes (7-9)).

Isolation of erythrocyte AK a in homogeneous form required a purification factor in excess of 18,000 times. Isolation of the minor enzyme forms at comparable purity would have required correspondingly larger amounts of starting material and was considered beyond the scope of the present study. The specific activity of the isolated AK a exceeded by severalfold apparent homogeneous preparations of the enzyme from various sources (1, 3-5) and was comparable to a preparation from squid muscle (2).

All of the isolated adenylate kinases examined thus far, including those of the human erythrocyte, share a common minimal molecular size of around 22,000 with concentration-dependent reversible association frequently encountered, leading to dimer (3, 5) and trimer stable enzyme forms (5). Tissue adenylate kinases exist in a number of electrophoretically distinguishable forms (23, 24) and present a variety of isoelectric points where examined (6, 25, 26). The erythrocyte enzymes display apparent anomalous electrophoretic behavior, migrating anodal to hemoglobin at pH 7.0 in starch gel (Fig. 1), while demonstrating isoelectric points considerably alkaline to hemoglobin by isoelectric focusing (Fig. 8) and cationic properties in relation to ion exchange celluloses. We find similar anodal electrophoretic migration in starch gel by rabbit muscle adenylate kinase and a pI value of 9.0 by isoelectric focusing (pI of 6.1 by moving boundary electrophoresis reported (25)). Anomalous electrophoretic behavior in starch gel has been observed with other enzymes (27, 28) in the presence of citrate buffers and attributed to charge modifications induced by binding of the polyanion.

Erythrocyte AK a and rabbit muscle adenylate kinase demonstrate a remarkable similarity in physicochemical and other properties including identical (a) thermal stability characteris-
ties, (b) substrate specificity, and (c) kinetic properties where compared. Kinetic studies with respect to the reaction mechanism remain limited by complexities involving enzyme and magnesium ion interaction.

REFERENCES