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PREPARATION AND CHARACTERIZATION OF A CRYSTALLINE HUMAN ATP:AMP PHOSPHOTRANSFERASE

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SUMMARY

1. An adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) has been purified 100-fold from human muscle with a final yield of 30 mg per kg of muscle.

2. Single crystals were grown from the electrophoretically homogeneous protein.

3. The specific activity was found to be 2000 μmoles of ATP produced or consumed per min per mg of protein at 25° and pH 8.0. The Michaelis constants for AMP, ADP, and ATP, respectively, are all in the range of 0.3 mM.

4. Studies on the physical properties indicate that the protein possesses a sedimentation constant of 2.30 S, a diffusion coefficient of $9.9 \cdot 10^{-7}$ $cm^2 \cdot sec^{-1}$, a partial specific volume of 0.74 ml/g and a mol. wt. of 21 500.

5. Amino acid analysis revealed a total of 194 residues, giving a calculated mol. wt. of 21 700. The composition was determined to be Asx_{13}, Thr_{13}, Ser_{12}, Glx_{26}, Pro_7, Gly_{18}, Ala_{10}, Val_{15}, Met_4, Leu_{17}, Ile_8, Tyr_7, Phe_5, Lys_{20}, His_4, Arg_{13}, Trp_0, Cys_2, amide $ammonia_{12}$.

INTRODUCTION

The concentrations of ATP, ADP, and AMP in most living cells seem to represent a system in true chemical equilibrium[1-3]. The reaction ATP + AMP $\rightleftharpoons$ 2 ADP is catalyzed by ATP:AMP phosphotransferases (EC 2.7.4.3) which are usually referred to as adenylate kinases. Only the classical adenylate kinase[4], the principal isozyme in muscle, is usually called myokinase. As judged from more than 200 publications[5] human adenylate kinases have attracted a good deal of attention in genetics, pharmacology, biochemistry, and clinical medicine, but none of these enzymes has been isolated so far.

* The studies reported in this paper were taken from a thesis submitted by E. Thuma to the Medizinische Fakultät der Ruperto-Carola-Universität in Heidelberg.

Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoate); PHMB, *p*-hydroxymercuribenzoate.

Adenylate kinases have been purified from rabbit muscle[6], porcine liver[7], bovine liver mitochondria[8], cockroach muscle mitochondria[9], baker's yeast[10], calf lens[11], rat liver organelles[12], porcine muscle[13], carp muscle[14] and squid muscle[15]. The preparative methods, however, were never found to be exchangeable from one source to another.

MATERIALS AND METHODS

Muscle

The muscles for the experiments described here were obtained from the legs of a 25-year-old man who had been seriously injured in a car accident. Immediately after the amputation of the limbs 3 kg of muscle were isolated and cooled to 0° before the preparation was started.

For preliminary experiments smaller amounts of muscle were collected over a period of 3 weeks. They were stored at −25° and allowed to warm up overnight at room temperature before use. These muscles were obtained from extremities which had to be amputated because of pathological conditions such as ischaemia, diabetic gangrene, or sarcoma. Only muscles which were apparently unaffected by a disease process were used for our experiments.

Chemicals and biologicals

Most reagent grade chemicals were purchased from Merck or Serva, dithiothreitol from Calbiochem. Urea was recrystallized from ethanol. Stock solutions of DTNB (EGA) were prepared according to ELLMAN[16]. PHMB was obtained from Serva and stock solutions were prepared and standardized as described by BOYER[17]. AMP, ADP and ATP (Boehringer) were stored at −20° as 0.012 M stock solutions of pH 6.8. Hexokinases (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), essentially free from adenylate kinase contamination, were obtained from Seravac or from Boehringer. The commercial hexokinase preparations were dialyzed exhaustively against 10^{-4} M EDTA (pH 6.0) and then diluted to 2000 enzyme units per ml with H_2O. These solutions were kept at −20° when not in use. Enzymes and chemicals for the standard colorimetric assay procedures of muscle adenylate kinase[18,19] were purchased from Boehringer.

Enzymic activity and protein concentration

For routine work adenylate kinase activity was measured according to the titrimetric pH-stat procedure described by MAHOWALD *et al.*[20] and KRESS *et al.*[21] using a Radiometer TTT 1a titrator equipped with a scale expander, an SBR 2b titrigraph and an SBU 1a syringe burette. For the determination of kinetic parameters the microcolumn method of NODA[22] and/or standard colorimetric assay procedures[18,19] were applied.

The protein concentration in crude fractions was determined by the colorimetric biuret procedure of GORNALL *et al.*[23]; a biuret factor of 35 mg per absorbance unit was used for all crude fractions. The methods for measuring the concentration of pure muscle adenylate kinase are described under RESULTS.

Chemical, physical and enzymic properties

The experimental details are given under RESULTS in the text or in the legends of the corresponding figures and tables. Molecular weight marker proteins were obtained from Sigma except for carp and rabbit muscle adenylate kinase, these were gifts from Dr. Noda, Dartmouth Medical School, N.H., USA.

Notes on phosphocellulose

Phosphocellulose (Whatman P 11, 7.4 mequiv/g) was washed with 0.5 M NaOH–0.5 M NaCl, rinsed with distilled water and converted into the H^+ form with 0.1 M HCl. It was equilibrated then with 0.01 M imidazole–HCl at pH 7.0. This cellulose was used both for batchwise and column procedures. A 2.5 cm × 35 cm column was poured from a slurry in the cold and washed with 500 ml of 0.01 M imidazole–HCl (pH 7.0). It must be emphasized that phosphocellulose when used for the preparation of rabbit muscle adenylate kinase and pork adenylate kinase should also be equilibrated and washed with 0.01 M imidazole–HCl and not with a 0.1 M buffer as originally stated[21].

RESULTS

Isolation of the enzyme

All steps were carried out at 4°. Contact of the preparation with metal was avoided when possible.

Fraction I. The muscles were excised directly after amputation, and blood was removed by washing in ice water. 3000 g of muscles were passed through a meat grinder and then mixed with 12 l of 0.01 M imidazole–HCl (pH 7.0). 800-ml batches of this mixture were homogenized in an MSE Ato mix for at least 1 min; shortening this procedure to 30 sec resulted in very low yield of adenylate kinase activity. The homogenate was allowed to stand for 20 min before centrifugation (MSE 6L, 2500 rev./min, 30 min, 4°). The reddish-orange supernatant had a volume of 10 510 ml.

Fraction II. To Fraction I 660 g of wet phosphocellulose (= 31.4 g dry weight) pretreated as described under MATERIALS AND METHODS was added; the mixture was stirred for 20 min, and then the phosphocellulose was precipitated by centrifugation (MSE 6L, 2500 rev./min, 5 min, 4°). The supernatant fluid was discarded. The six 1-l centrifuge cups were used twice so that each of them eventually contained about 100 g of wet phosphocellulose. In order to remove contaminating proteins the phosphocellulose in each cup was washed 3 times with 0.01 M imidazole–HCl (pH 7.0) and then twice with 250 ml of 0.1 M imidazole–HCl (pH 7.0). The adenylate kinase was eluted with a total volume of 800 ml of 0.1 M imidazole–HCl–0.3 M NaCl; the cellulose was removed by centrifugation. Fraction II was pink.

Fraction III. To Fraction II solid $(NH_4)_2SO_4$ was added to 50% saturation (= 298 g per l solution)[6]. The mixture was stirred for at least 1 h, then 20 ml of 2 M HCl was added rapidly in order to bring the apparent pH to 3.0 within 1 min. After further 10 min of stirring 2 M NaOH was added slowly over a period of about 20 min until an apparent pH of 7.0 was reached; the suspension was then allowed to stand for 20 min. Centrifugation (MSE 6L, 2500 rev./min, 30 min, 4°) yielded a beige supernatant fluid, Fraction III, the $(NH_4)_2SO_4$ concentration of which was about 1.95 M (= 48% saturation).

Fraction IV. 279.6 g of $(NH_4)_2SO_4$ per l solution was added to Fraction III in order to raise the $(NH_4)_2SO_4$ concentration to 3.6 M (= 90% saturation). The suspension was stirred for at least 1 h, allowed to stand for 2–12 h, and then centrifuged (MSE 18, 12 000 rev./min, 30 min, 4°). The pellet was dissolved in about 70 ml of 0.01 M imidazole–HCl (pH 7.0), and freed from $(NH_4)_2SO_4$ by flow dialysis against 25 l of the same buffer. After a clearing centrifugation (MSE 18, 4°, 12 000 rev./min, 30 min) the protein solution was chromatographed on a 2.5 cm × 35 cm phosphocellulose column (for details see legend to Fig. 1). The fractions representing the major activity peak were combined to give the yellowish Fraction IV.

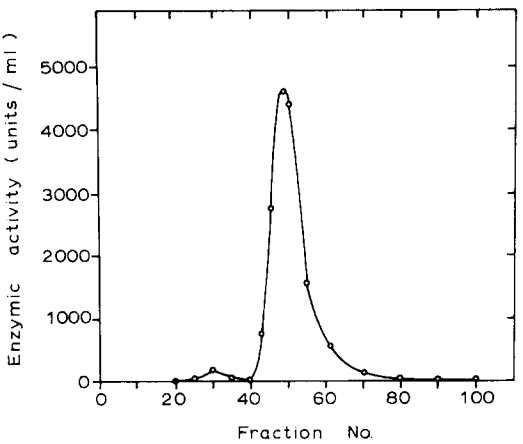

Fig. 1. Chromatography of muscle adenylate kinase on a phosphocellulose column at 4°. The 2.5 cm × 35 cm column and the protein solution (approx. 950 mg in 75 ml 0.01 M imidazole–HCl, pH 7.0) were prepared as described under MATERIALS AND METHODS. The sample was applied and the column washed with 1 l of 0.1 M imidazole–HCl (pH 7.0). Then 0.12 M imidazole–HCl–5 mM AMP (pH 7.0) was used as an eluant and the collection of 10-ml fractions started simultaneously. Adenylate kinase activity was found in a minor peak (15 mg of protein) and in a major peak containing 175 mg of protein. A flow rate of 100 ml/h was maintained with a peristaltic pump. Because of the presence of AMP the ultraviolet absorption was not measured.

Fraction V. On adding 614 g of $(NH_4)_2SO_4$ per l to Fraction IV the protein was precipitated. It was then dissolved in 20 ml of 0.1 M imidazole–HCl (pH 7.0), applied to a 4 cm × 130 cm column of Sephadex G-75, and eluted with the same buffer at a flow rate of 45 ml/h; 5-ml fractions were collected. The elution volume for myokinase was about 800–1100 ml. Fractions with a specific activity higher than 1800 units/mg were collected to give Fraction Va. The adjacent fractions with a specific activity ranging from 1000–1800 units/mg were combined, precipitated with $(NH_4)_2SO_4$ and reapplied to the Sephadex G-75 column. The adenylate kinase activity (Fraction Vb) was eluted in a symmetrical peak which was well separated from a reddish protein of molecular weight 30 000.

The purification procedure is summarized in Table I. The final product, the protein from Fractions Va and Vb, appeared homogeneous as judged from polyacrylamide gel electrophoresis under various conditions (Fig. 2).

TABLE I

PURIFICATION OF HUMAN MUSCLE ADENYLATE KINASE (INITIALLY 3000 g OF MUSCLE)

Step	*Resulting fraction*	*Vol. (ml)*	*Total protein (mg)*	*Total units (units/ fraction)*	*Specific activity (units/mg)*	*Purification (-fold)*	*Recovery (%)*
1. Extraction of muscle	I	10 470	32 200	620 000	19.2	1	100
2. Fractionation* with phosphocellulose	II	1 690	1 950	362 000	184	9.5	58.4
3. $(NH_4)_2SO_4$ and pH fractionation	III	1 880	985	286 000	290	15.1	46.1
4. Chromatography* on phosphocellulose	IV	290	174	220 000	1260	65.1	35.5
5. Gel filtration on Sephadex G-75	Va	28	47	104 000	2200	114.5	16.8
	Vb	35	50	96 000	1920	100	15.5

* Although we purified only the quantitatively predominating adenylate kinase in muscle in the course of our preparations there were regular indications of the presence of isozymes[37]. A minor component with adenylate kinase activity is shown in Fig. 1, another one seems to have been bound tightly to the phosphocellulose in Step 2 and could be washed off only with 0.1 M imidazole–0.6 M NaCl (pH 7.0).

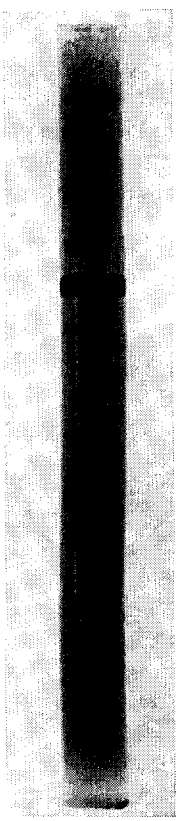

Fig. 2. Gel electrophoresis of human muscle adenylate kinase. 100 μl of enzyme stock solution was added to 900 μl of 5.5 M urea–2 M acetic acid. 5 μl of this solution containing 8 μg of protein was submitted then to electrophoresis in gels of 7.5% acrylamide and 0.2% methylene bisacrylamide containing 5 M urea at pH 4.3. Electrophoresis was carried out at room temperature for 3 h at 4 mA per tube, origin at top, cathode at bottom. The gels were stained with Coomassie brillant blue and destained in 10% acetic acid–7% methanol.

Gravimetric standardization of the protein stock solution

The protein of Fraction Va (50 mg) was precipitated by adding 0.6 g of solid $(NH_4)_2SO_4$ per ml. It was collected by centrifugation (MSE 18, 12 000 rev./min, 20 min, 4°), dissolved in 3 ml of 0.1 M Tris–HCl–2 mM dithiothreitol (pH 8.0) and dialyzed 3 times against 1000 vol. of distilled water. After a clearing centrifugation (MSE 18, 12 000 rev./min, 20 min, 4°) the solution was stored at 4°. It was found to be stable for 6 weeks at this temperature and was used as a stock solution for all experiments.

750 μl of the stock solution was dried by lyophilization to a constant weight (12.08 $\pm$ 0.02 mg in a tared 2 ml flask). The amount of protein-bound water was neglected since a further drying procedure at 120° and 10 mm Hg did not reduce the weight of the sample. Hence the protein concentration of the stock solution was assumed to be 16.1 mg/ml. This value was used for determining the ultraviolet absorption indices, the biuret factor[20] and the refractive index increment for human adenylate kinase. The results are given in Table IV.

Preparation of single crystals

500 μl of the protein stock solution (16.1 mg/ml) was dialyzed against 1 l of 0.15 M imidazole–HCl–1.8 M $(NH_4)_2SO_4$ (pH 6.9). After dialysis the volume of the protein solution was brought to 500 μl by adding dialysis fluid, and it was then mixed with 250 μl of 0.15 M imidazole–HCl–4.0 M $(NH_4)_2SO_4$ (pH 6.9). After 10 days at 4° single crystals had grown in the turbid solution (Fig. 3). These crystals look very similar to those of porcine muscle adenylate kinase which are being used for X-ray analysis[13].

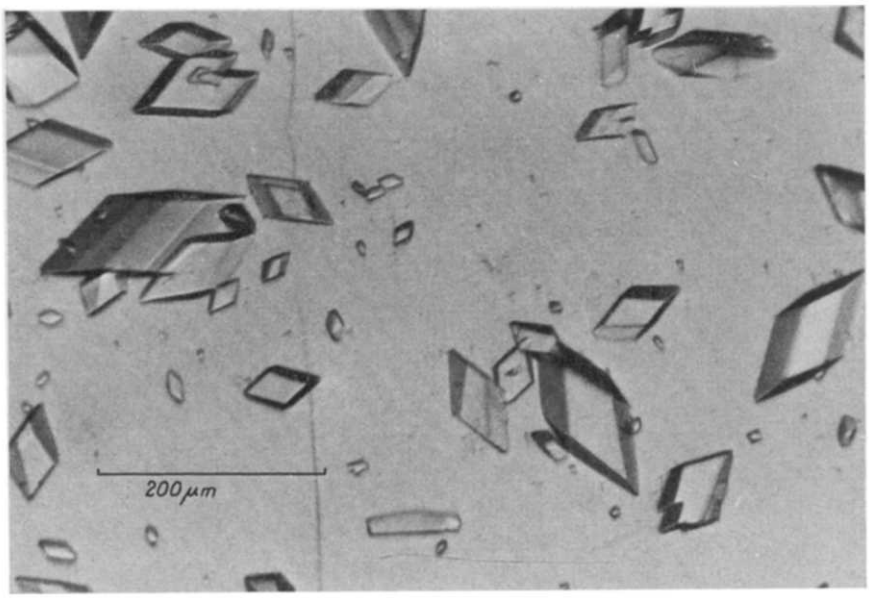

Fig. 3. Human muscle adenylate kinase crystals in partially polarized light.

Determination of physicochemical constants

For the estimation of the molecular weight three methods were used. By gel filtration[24] on Sephadex G-75 and G-100 a value of 21 500 was determined (Fig. 4). Dodecyl sulfate gel electrophoresis[25] yielded the same result (Fig. 5). Under the assumption that human muscle adenylate kinase contains two readily titratable SH groups, as in the case of the analogous proteins from pig[13] and rabbit[21,26], the molecular weight can be estimated by determining the thiol content of the enzyme. Using ELLMAN's[16] method $8.8 \cdot 10^{-8}$ moles of cysteine per mg of human adenylate kinase were obtained; $8.9 \cdot 10^{-8}$ moles of cysteine per mg of protein were found by titrating the protein with PHMB[17,21]. This corresponds to a value of 2 moles of sulfhydryl groups per 22 600 g of protein.

The value of the partial specific volume was estimated from the total amino acid composition according to the method of COHN AND EDSALL[27].

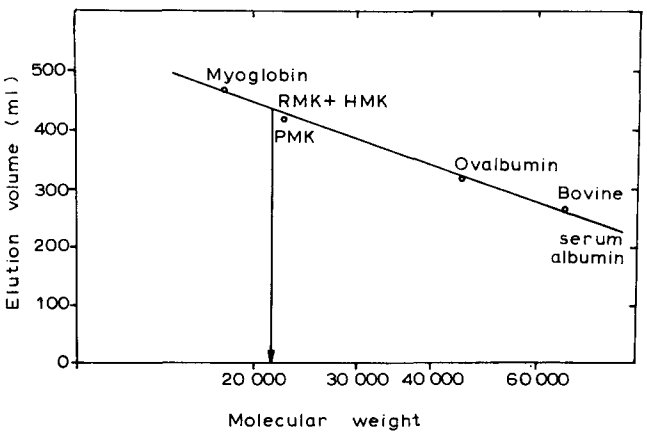

Fig. 4. Molecular weight determination by gel filtration on Sephadex G-75 (cf. ref. 24). HMK, human muscle adenylate kinase; RMK, rabbit muscle adenylate kinase; PMK, porcine muscle adenylate kinase.

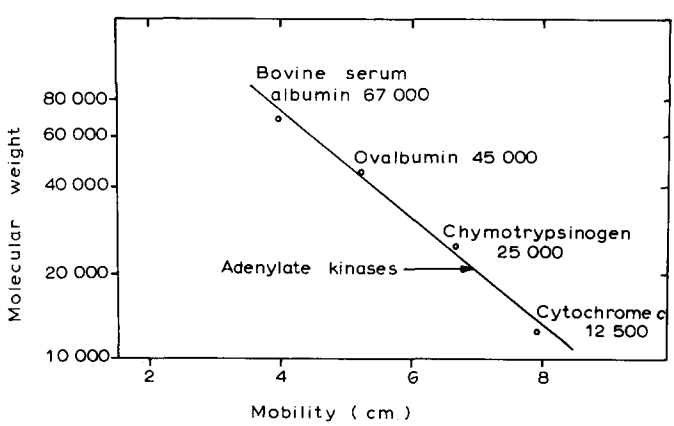

Fig. 5. Molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis (*cf.* ref. 25). Human muscle adenylate kinase and porcine muscle adenylate kinase (= adenylate kinases) showed identical mobilities.

For determining the diffusion constant the gel filtration method described in detail by ACKERS[28] was followed. In our experiments the only reference protein was rabbit muscle adenylate kinase (Table II). Since the elution volumes for the two proteins were practically identical in all cases it follows that their Stokes' radii and consequently their diffusion coefficients are also identical. From the data given by

TABLE II

DETERMINATION OF THE DIFFUSION COEFFICIENT BY GEL FILTRATION (*cf.* REF. 28)

In each experiment 1 mg of protein (= 2000 enzyme units) was submitted to gel filtration at 20°. The eluant was 0.15 M KCl–0.01 M potassium phosphate (pH 7.0). The elution volumes for rabbit or human adenylate kinase were determined by measuring the enzymatic activity.

Sephadex type and total volume V_t of the column	*Elution volume (ml)*	
	Rabbit muscle adenylate kinase	*Human muscle adenylate kinase*
G-75 (V_t = 720 ml)	435	434
G-100 (V_t = 226 ml)	150	152
G-200 (V_t = 140 ml)	127	126

TABLE III

AMINO ACID COMPOSITION OF HUMAN MUSCLE ADENYLATE KINASE

Except for cysteine the amino acid composition of human adenylate kinase was determined according to procedures which have been described in great detail for rabbit muscle adenylate kinase[26]. Four samples, each containing 0.75 mg of adenylate kinase in 1 ml of constant-boiling HCl–1% phenol, were hydrolyzed in sealed evacuated tubes at 110° for 20, 40, 70 and 140 h, respectively. After removal of the HCl in a desiccator over NaOH pellets amino acid analyses were carried out with a Locarte automatic amino acid analyzer. The results given in the table were corrected for Ser, Thr, Val, Ile, Leu, and for ammonia. Trp analyses were performed after $Ba(OH)_2$ hydrolysis[26].

Amino acid residue	*Number of amino acid residues in 21 500 g of protein*	*Nearest integral number of amino acid residues for 21 500 and 21 686 g of protein*	*Integral number of residues × respective residue molecular weight*
Aspartic acid	12.74	13	1496.0
Threonine	13.08	13	1314.3
Serine	12.03	12	1044.8
Glutamic acid	25.71	26	3356.1
Proline	6.84	7	679.8
Glycine	18.09	18	1027.1
Alanine	9.65	10	710.7
Valine	14.76	15	1487.0
Methionine	3.72	4	524.8
Isoleucine	7.68	8	905.2
Leucine	16.83	17	1923.6
Tyrosine	6.75	7	1142.3
Phenylalanine	4.97	5	735.9
Lysine	19.62	20	2563.4
Histidine	3.80	4	548.7
Arginine	13.17	13	2030.3
Amide ammonia	12.1	12	−10.8
Cysteine	1.89	2	206.3
Tryptophan	0.12	0	—
Total		194	21686

NODA AND KUBY[29] a $D^{0\%}_{20^\circ}$ of $9.9 \cdot 10^{-7}$ $cm^2 \cdot sec^{-1}$ can be calculated for rabbit muscle myokinase.

The sedimentation coefficient was calculated according to SVEDBERG's[30] equation. Values of 0.74 ml/g, 21 500 and $9.9 \cdot 10^{-7}$ $cm^2 \cdot sec^{-1}$ were used for the partial specific volume, the molecular weight, and the diffusion coefficient, respectively.

Amino acid analysis

Only one enzyme preparation—representing the adenylate kinase of one individual man—was used for the analysis. Therefore the results given in Table III must be interpreted with appropriate reservations.

TABLE IV

MOLECULAR PROPERTIES OF HUMAN MUSCLE ADENYLATE KINASE

Property	*Value*	*Method*
1. Molecular weight	(a) 21 700	Amino acid analysis[26].
	(b) 22 600	Titration of two SH groups[21,26].
	(c) 21 500	Dodecyl sulfate polyacrylamide gel electrophoresis[25].
	(d) 21 500	Gel filtration on Sephadex G-75 (ref. 24)
2. Diffusion coefficient $D^{0\%}_{20^\circ}$ ($cm^2 \cdot sec^{-1}$)	$9.9 \cdot 10^{-7}$	Gel filtration[28]
3. Specific volume $\bar{v}_{20^\circ}$ ($ml \cdot g^{-1}$)	0.74	Amino acid analysis[27]
4. Sedimentation constant $s^0_{20^\circ}$ (sec)	$2.3 \cdot 10^{-13}$	Calculated by use of the data of 1a, 2, and 3
5. Frictional ratio f/f_0	1.15	Calculated by use of the data of 1a, 2, 3, and 4
6. Specific absorbance* $A^{1\%}_{1\,cm}$ at $\lambda = 279$ nm	6.67	**
7. Molar absorbance* at $\lambda = 279$ nm	14 300	**
8. Biuret factor (10 ml total volume, 1 cm light path, $\lambda = 540$ nm) (mg/absorption unit)	32.0	**
9. Refractive index increment $(\Delta n/\Delta c)_{25^\circ}$ for $\lambda = 589$ nm ($g/100\ ml)^{-1}$	$1.81 \cdot 10^{-3}$	**

* The molar absorbance is not in agreement with the values reported for rabbit adenylate kinase (11 300)[29] and for porcine adenylate kinase (11 500)[13]. This phenomenon, the only significant physicochemical discrepancy between the three analogous enzymes, must be reinvestigated. Our preliminary findings indicate that the $A_{280\ nm}/A_{260\ nm}$ ratio and the number of tyrosine residues are identical for the three proteins.

** Gravimetric standardization.

Determination of kinetic constants

For ease of comparison the conditions and techniques used by NODA[22] for rabbit muscle adenylate kinase were applied (Table V). The K_m for ADP is still a matter of controversy[31] and should be reinvestigated by taking into account that ADP and MgADP may be two different substrates.

TABLE V

KINETIC PROPERTIES OF HUMAN MUSCLE ADENYLATE KINASE

The conditions used for the enzyme catalyzed reactions were: $T = 25°$; 0.05 M Tris–HCl, (pH 8.0); 1 μg of adenylate kinase in 10 ml total volume.

	Forward reaction	*Backward reaction*
Reaction	2 ADP → ATP + AMP (MgADP + ADP → MgATP + AMP), *cf.* ref. 31	ATP + AMP → 2 ADP (MgATP + AMP → MgADP + ADP), *cf.* ref. 31
Assay	(a) Microcolumn technique[22] (b) Coupled enzyme systems (colorimetric[18,19] and pH-titrimetric[20] procedures)	(a) Microcolumn technique[22] (b) Coupled enzyme system (colorimetric procedure[18])
Optimal $[Mg^{2+}]$	$[Mg^{2+}] = \frac{1}{2}$ [ADP]	$[Mg^{2+}]$ = [ATP]
Michaelis constant (K_m)	0.35 mM for ADP	0.32 mM for AMP at [ATP] = 3 mM; 0.27 mM for ATP at [AMP] = 3 mM
Molecular activity	47 000 units/μmole enzyme	41 000 units/μmole enzyme
Specific activity	2200 units/mg protein	1900 units/mg protein

DISCUSSION

The subunits of all known adenylate kinases[6–15] as judged from their molecular properties and their amino acid compositions seem to be equivalent proteins. Therefore these ubiquitous enzymes might help in establishing the family tree of living organisms[32] and the relationship between subcellular structures[33,34].

More practical problems prompted us to characterize the human enzyme. The investigation of adenylate kinases could be an aid in finding the basic biochemical lesion of progressive muscular dystrophy[35]. Moreover adenylate kinases might prove useful as drugs for desintegrating blood platelet thrombi[36]. The enzyme described here has certain desirable features as a pharmacon: it is homogeneous, has a sufficiently low Michaelis constant for ADP and a high specific activity; it can be lyophilized and redissolved without loss of activity, and its half-life in the plasma is in the range of 3 to 6 h (R. H. SCHIRMER, unpublished results).

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