Partial Purification and Properties of the Two Common Inherited Forms of Human Erythrocyte Adenylate Kinase

By C. BROWNSON* and N. SPENCER

Department of Biochemistry, University of London King's College, Strand,
London WC2R 2LS, U.K.

(Received 5 June 1972)

1. The partial purification of adenylate kinase, types 1 and 2, from human erythrocytes is described. 2. Gel chromatography of both forms of the enzyme gave estimates of the molecular weights in the range 20000–23000. 3. Studies on crude haemolysates at various pH values indicated that the type 2 enzyme was less stable than the type 1. Heat denaturation studies on the partially purified enzymes confirmed these findings. 4. Measurements of rates of inhibition by iodoacetate and iodoacetamide showed that the type 2 enzyme reacts more readily than the type 1 enzyme with both reagents. 5. The effect of temperature on the initial velocity of ADP formation was measured at a single concentration of both AMP and MgATP^{2-} . The two forms of the enzyme responded differently to increasing temperature.

Fildes & Harris (1966) showed that the enzyme adenylate kinase (EC 2.7.4.3) exists in different inherited forms in the erythrocytes of different individuals. Two forms of the enzyme could be distinguished by their electrophoretic mobility in starch gel and these were designated types 1 and 2. Family studies indicated that the two forms of the enzyme are controlled by two allelic genes and an extensive population survey revealed the three types of individuals expected on this basis: namely two homozygotes designated types 1 and 2 whose erythrocytes contained only the type 1 or type 2 enzymes respectively and heterozygous individuals, type 2–1, whose erythrocytes contained both forms of the enzyme.

The two forms of the enzyme appear to be associated with small but significant differences in the total amount of catalytic activity of adenylate kinase in erythrocytes from individuals of different phenotype. Rapley & Harris (1970) reported that the total activity of the enzyme in haemolysates prepared from erythrocytes of type 1 individuals was slightly higher than that found in haemolysates of erythrocytes from type 2–1 individuals. In another comparative study Modiano *et al.* (1970) reported a similar but more marked difference in total adenylate kinase activity between these two phenotypes. This finding may reflect either differences in amount of the two enzyme proteins or differences in their catalytic properties.

In the present work we report the partial purification of the two common forms of erythrocyte

adenylate kinase, together with the results of preliminary studies on some of the properties of the enzymes particularly those relating to the stability of the enzyme. In the accompanying paper (Brownson & Spencer, 1972) we describe detailed studies of the catalytic mechanism of the enzymes.

Experimental

Materials

Chemicals. Enzymes and substrates used in the linked assay system were obtained from Boehringer Corp., London, W.5., U.K. Hydrolysed starch was obtained from Connaught Medical Laboratories, Toronto, Ont., Canada. Iodoacetic acid and iodoacetamide were twice recrystallized from light petroleum (b.p. 60–80°C) and ethanol respectively. All other chemicals were AnalaR grade or the best preparations commercially available.

Buffers. All pH measurements were done at room temperature. Anionic buffers were prepared by adding NaOH to the appropriate acid or acid salt to give the required pH and molarity of anion. Similarly cationic buffers were prepared by using the free base and HCl.

Methods

Starch-gel electrophoresis of adenylate kinase. Starch gels (16%, w/v) were prepared in 5 mM-sodium succinate buffer, pH 5.0, and 100 mM-succinate buffer of the same pH was used in the electrode compartments. Gels were run for 16 h at 3 V/cm in the cold

* Present address: Department of Biochemistry, Guy's Hospital, London E.C.1, U.K.

room. Under these conditions all the adenylate kinase components migrated towards the cathode; otherwise the procedure for running and staining the gels for adenylate kinase activity was exactly as described by Fildes & Harris (1966).

Molecular-weight determinations. Approximate molecular weights were determined by gel filtration and sucrose-gradient centrifugation. Gel filtration was done by the method of Andrews (1965). Sephadex G-75 (Pharmacia, Uppsala, Sweden) was swollen in 10 mM-sodium citrate buffer, pH 6.0, and packed into a column (2.5 cm $\times$ 100 cm). The following proteins were used as standards: cytochrome *c* (mol.wt. 12400); horse myoglobin (mol.wt. 17800); bovine serum albumin (mol.wt. 68000) and chymotrypsinogen (mol.wt. 25000).

Sucrose-gradient centrifugation was done essentially as described by Martin & Ames (1961). Gradients were layered by hand using 5, 10, 15 and 20% (w/v) solutions of sucrose in 10 mM-sodium citrate buffer, pH 6.0. Solutions (1.2 ml) were carefully layered on to the surface of the preceding denser layer in cellulose acetate Spinco tubes which were then left at 4°C for 2 to 6 h to equilibrate. Just before centrifugation the sample (0.1 ml) was mixed with lysozyme (0.1 ml, 10 mg/ml) in 10 mM-citrate buffer, pH 6.0, and carefully layered on to the surface of the gradient. Centrifugation was done in a Spinco model L ultracentrifuge pre-cooled to 5°C using a swinging bucket rotor (SW 50) at 50000 rev./min for 16 h. At the end of the run the bottom of the centrifuge tube was pierced with a fine needle and approx. 21 fractions (8 drops/fraction) were collected from each tube and analysed for adenylate kinase activity, lysozyme activity and protein concentration. From the relative migration of adenylate kinase and lysozyme in the gradient a rough estimate of the molecular weight of adenylate kinase was obtained.

Lysozyme activity. Activity was detected by measuring the decrease in E_{650} of a suspension of cell walls of *Micrococcus lysodeikticus* in 10 mM-tris-HCl buffer, pH 8.0, adjusted to give an E_{650} of 1.5.

Adenylate kinase activity. This was measured in the direction 2 ADP $\rightarrow$ AMP + ATP by using the coupled assay system of Oliver (1955). Measurements were made with a Unicam SP. 800 spectrophotometer at 30°C. Reactions were done in a total volume of 1 ml using semi-micro cuvettes with 1 cm light-path. The final concentrations of the reaction components were as follows: tris-HCl, pH 8.0, 100 mM; ADP (trisodium salt), 1 mM; glucose, 40 mM; NADP⁺ (disodium salt), 180 μ M; MgCl₂, 2 mM; glucose 6-phosphate dehydrogenase, 0.25 units; hexokinase, 0.5 units. The reaction was initiated by the addition of adenylate kinase and the steady state was usually reached within 2 min. Reaction rates were proportional to the amount of adenylate kinase added provided the extinction change did not exceed 0.03/min. Adenylate

kinase activity is expressed as units/mg of protein, or, for column and centrifuge fractions, as units/ml of fraction. One unit corresponds to 1 μ mol of ATP produced/min.

Protein concentration. In crude haemolysates protein determinations were based on haemoglobin which was determined by the method of Crosby *et al.* (1954). In solutions which were essentially free of haemoglobin, protein was determined by the microtechnique of Lowry *et al.* (1951) at a wavelength of 500 nm with bovine serum albumin (Armour Laboratories Ltd., Eastbourne, Sussex, U.K.) as a standard. For column and centrifuge fractions the E_{280} was used as an approximate measure of protein concentration.

Partial purification of adenylate kinase from human erythrocytes. The erythrocytes used in the present work were separated from whole blood collected into acid-citrate-dextrose solution (Drabkin, 1949). The whole blood samples were obtained from two main sources: Pharmacology Department, Royal College of Surgeons, London W.C.2, U.K., and Regional Blood Transfusion Service, Tooting, London S.W.17, U.K.

After centrifugation at 3000 rev./min for 10 min plasma and leucocytes were removed by aspiration. The erythrocytes were gently resuspended in 0.15 M-NaCl and washed by centrifugation at 3000 rev./min for 10 min. This procedure was repeated twice with fresh saline. After the final wash the supernatant saline solution was removed as completely as possible and the erythrocytes were lysed by the addition of two volumes of water containing 100 μ M-dithiothreitol. All solutions used in subsequent purification procedures contained 100 μ M-dithiothreitol. Stroma were precipitated by carefully adjusting the pH of the haemolysate to 6.0 with 1 M-HCl and centrifuging at 71000 rev./min for 30 min. The supernatant was used in all experiments involving crude haemolysates and for further purification steps. Crude haemolysate (50 ml), dialysed for 24 h against frequent changes of 10 mM-sodium citrate buffer, pH 5.0, was applied to a column (2.5 cm $\times$ 50 cm) of CM-Sephadex (Pharmacia Ltd., London, W.13, U.K.) previously equilibrated against the same buffer. The column was eluted with a linear gradient of NaCl (100–250 mM) in the same buffer. Fractions (approx. 15 ml) were collected by using an automatic timer. Those fractions containing adenylate kinase were pooled and concentrated by pressure dialysis using an Amicon Diaflo ultrafiltration cell (Amicon, High Wycombe, Bucks., U.K.) with a UM-10 filter. The concentrated fractions (see the Results section) from four such columns were pooled and dialysed against several changes of 10 mM-citrate buffer, pH 5.0, for 24 h and then applied to a second CM-Sephadex column (1.5 cm $\times$ 20 cm) equilibrated against the same buffer. The column was eluted with 150 mM-NaCl in the same buffer. The

fractions containing adenylate kinase activity were combined and concentrated by pressure dialysis.

Results

Purification

Fig. 1 shows the elution pattern obtained when a crude haemolysate prepared from erythrocytes of an individual of adenylate kinase type 1 was chromatographed on the first CM-Sephadex column. Two peaks were consistently found. The proportion of total adenylate kinase activity represented by the minor peak increased with increasing age of the haemolysate used in the separation, or with the duration of storage of the blood from which haemolysates were prepared. Haemolysates prepared from freshly taken blood used immediately gave a minor

Fig. 1. Chromatography of a crude type 1 haemolysate on the first CM-Sephadex column

Experimental conditions are described in the text.
●, Adenylate kinase activity; ○, protein.

enzyme peak representing 0–5% of the total adenylate kinase activity. When the erythrocytes were stored for two weeks in acid–citrate–dextrose solution at 5°C and then haemolysed, or when haemolysates were similarly stored, up to 25% of the total enzyme activity was found in the minor enzyme peak. Material from both peaks was further investigated by density-gradient centrifugation. The minor peak from the CM-Sephadex column showed two components with approximate molecular weights of 20000 and 29000, whereas the major peaks showed only one component of molecular weight approximately 19000. Since the molecular weight of the enzyme was found to be about 23000, by gel chromatography in the present work it seems probable that the minor peak eluted from the column contains adenylate kinase complexed with a small protein of molecular weight 6000–10000. The major and minor fractions of enzyme activity gave identical patterns on starch-gel electrophoresis.

Haemolysates prepared from erythrocytes of an individual of type 2 constitution gave similar elution patterns on the first CM-Sephadex column. Both forms of the enzyme were eluted as single symmetrical peaks from the second CM-Sephadex columns. Table 1 shows a typical purification scheme for a type 1 haemolysate.

Molecular weights

Molecular weights determined by gel chromatography of crude haemolysates and partially purified enzyme preparations were in the range 20000–23000. Similar values were obtained from the results of analytical centrifugation. Both forms of the enzyme gave essentially identical estimates. These values are in agreement with those reported by Rapley (1969) and by Bowman *et al.* (1967).

Table 1. Purification of adenylate kinase from type 1 erythrocytes

For details see the text.

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Purification factor	Yield (%)
Crude haemolysate	20	412	18200	0.226		100
First CM-Sephadex column: fractions of main enzyme peak combined and concentrated by pressure dialysis	8.4	300	11.9	25.2	113	72.8
Second CM-Sephadex column: fractions having adenylate kinase activity concentrated by pressure dialysis	2.5	89	1.5	60	266	21.6

Fig. 2. Heat denaturation of partially purified adenylate kinase types 1 (●) and 2 (○)

Experimental conditions are described in the text.

sodium phosphate buffer, pH7, containing 1mM-5,5'-dithiobis-(2-nitrobenzoic acid) at 20°C. Almost 100% of the original activity could be regenerated by immediate treatment with 1mM-dithiothreitol.

In contrast, at 20°C iodoacetate and iodoacetamide did not inhibit the enzyme, but at 42°C the inhibition was measurable and followed first-order kinetics with respect to time. In these experiments the enzyme was incubated in a reaction mixture containing 50mM-sodium phosphate, pH7, and albumin (10mg/ml). The inhibitor concentration was varied as required for each experiment. The reaction was started by placing the reaction mixture in a water bath at 42°C. Samples were removed at appropriate intervals and immediately assayed for activity. Concentrations of iodoacetate and iodoacetamide used in these experiments did not affect the enzymes present in the linked-assay system. The course of the inhibition was followed until at least 50% of the initial adenylate kinase activity had disappeared. Loss of activity through heat denaturation was negligible under these conditions. Duplicate runs were done at each concentration of inhibitor which was tested. Pseudo-first-order rate constants were obtained from plots of the logarithm of percentage residual activity against time. From the slopes of plots of pseudo-first-order rate constants against concentration of iodoacetamide the apparent second-order constants for the inhibition by iodoacetamide were obtained (Fig. 3).

Fig. 3. Pseudo-first-order rate constants for the inhibition of adenylate kinase types 1 (■) and 2 (□) at different concentrations of iodoacetamide

Experimental conditions are described in the text.

These were 6.8 and 10.2 litre·mol⁻¹·min⁻¹ for the type 1 and type 2 enzymes respectively.

Inhibition by iodoacetate was tested under the same conditions. The pseudo-first-order rate constants for inhibition by iodoacetate (1mM) were 0.0016 and 0.0027 min⁻¹ for adenylate kinase types 1 and 2 respectively. The corresponding constants for inhibition by iodoacetamide (1mM) were 0.0069 and 0.0091 min⁻¹ respectively. The relative reactivity of the two enzymes towards iodoacetate (1mM) is proportional to the ratio of the appropriate rate constants, i.e. 1.68. A similar value is obtained for the relative reactivity with iodoacetamide (1mM), namely 1.32. The similarity of these ratios suggests that both inhibitors react with the same group in each enzyme and that this group occupies a similar position in both enzymes.

Fig. 4. Effect of temperature on the initial velocity of the adenylate kinase reaction measured in the direction $AMP + ATP \rightarrow 2 ADP$

●, Adenylate kinase type 1; ○, adenylate kinase type 2. Experimental details are given in the following paper (Brownson & Spencer, 1972).

Effect of temperature on initial velocity

We have made a preliminary study of the effect of temperature on the reaction catalysed by the two forms of the enzyme since heats of activation possibly may be correlated with biological effectiveness (Wroblewski & Gregory, 1961). It was not feasible to use linked assay systems in these studies because of the possible effects of heat on the constituent enzymes in these systems. We therefore used the radiochemical assay system described in the following paper (Brownson & Spencer, 1972). Initial velocities of the reverse reaction were measured at several temperatures with fixed concentrations of the two substrates, namely 1 mM-AMP and 1 mM-MgATP²⁻ (subsequently referred to as 'MgATP'). Duplicate determinations were made on each enzyme at temperatures over the range 25° to 45°C.

The concentrations of both substrates were not saturating so it was not possible to correlate the initial velocity values obtained with a particular rate constant. However, as Fig. 4 shows, under the conditions tested, the two forms of the enzyme respond differently to changes in temperature.

Discussion

Few studies have been published previously on human erythrocyte adenylate kinase. The value reported here for the molecular weight of the enzyme is in agreement with the values obtained by Rapley (1969) and by Bowman *et al.* (1967). In contrast with Rapley (1969) we found a marked difference in stability between the type 1 and type 2 enzymes. Measurements of thermostability on partially purified enzymes are subject to uncertainty because of the wide variety of factors that are known to affect enzyme stability. However, in the present work evidence obtained by using two different approaches indicates that the type 2 enzyme is less stable than the type 1 enzyme. Culliford (1971) reported that the type 2 enzyme was more sensitive to ageing than the type 1 enzyme.

Little is known about the processes of enzyme degradation *in vivo* but it has been suggested that with two erythrocyte enzymes, namely catalase (Aebi, 1967) and glucose 6-phosphate dehydrogenase (Yoshida *et al.*, 1967; Luzzatto & Allan, 1965) the stability of certain inherited forms of these enzymes *in vitro* may be correlated with their corresponding stability *in vivo*. A similar correlation could possibly account for the lower total amount of adenylate kinase activity measured in erythrocytes from type 2-1 individuals compared with the amount found in erythrocytes of type 1 individuals.

The greater reactivity of the type 2 compared with the type 1 enzyme towards iodoacetate and iodoacetamide may possibly be correlated with the decreased stability of the type 2 enzyme, since studies on adenylate kinase obtained from other sources (see references under 'Inhibitor studies' above) suggest that thiol groups are involved in maintaining the enzyme in an active conformation.

Classical studies on the reaction of iodoacetate with ribonuclease illustrate the dangers of assuming that this inhibitor necessarily reacts with thiol groups in proteins (cf. Zittle, 1946; Stein & Barnard, 1959). However, the fact that both erythrocyte enzymes are inhibited by 5,5'-dithiobis-(2-nitrobenzoic acid) does indicate the presence of thiol groups in the enzymes and suggests that iodoacetate and iodoacetamide may react with the same thiol group. This question will only be satisfactorily resolved when the pure enzymes become available so that the amino acid residues that are modified by reaction with iodoacetate or iodoacetamide may be identified unequivocally.

Studies on the effect of heat on the initial velocities of the reaction catalysed by the two erythrocyte enzymes were done at non-saturating concentrations of substrate. In the case of bi-substrate reactions several rate constants may be modified by increasing temperature. However, assuming that the same rate constant(s) is affected by increased temperature in the

two forms of the enzyme the results suggest that for the reverse reaction with MgATP and AMP as substrates the type 2 enzyme has a lower energy of activation. Presumably the greater catalytic efficiency that this implies could compensate for the unfavourable stability characteristics of the type 2 enzyme.

The authors thank D. Mills, Royal College of Surgeons, and Dr. J. Vincent Barry of the South London Transfusion Centre for supplies of human blood. C. B. gratefully acknowledges the receipt of a Wellcome Research Training Scholarship. This work represents part of a thesis submitted for the degree of Ph.D. at the University of London by C. B.

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