Kinetic Studies on the Two Common Inherited Forms of Human Erythrocyte Adenylate Kinase

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1. The kinetic properties of two genetic variants of human erythrocyte adenylate kinase were studied at limiting concentrations of both ADP and MgADP^- in the forward direction and at limiting concentrations of both AMP and MgATP^{2-} in the reverse direction. 2. Primary reciprocal plots rule out the possibility of a Ping Pong mechanism for both forms of the enzyme. 3. Analysis of the kinetic data by an appropriate computer program gave the following K_m values for the type 1 enzyme: AMP, $0.33\text{ mM} \pm 0.1$; MgATP^{2-} , $0.95\text{ mM} \pm 0.13$; ADP, $0.12\text{ mM} \pm 0.03$; MgADP^- , $0.22\text{ mM} \pm 0.04$. Values for the type 2 enzyme were: AMP, $0.27\text{ mM} \pm 0.03$; MgATP^{2-} , $0.40\text{ mM} \pm 0.05$; ADP, $0.08\text{ mM} \pm 0.07$; MgADP^- , $0.20\text{ mM} \pm 0.04$. 4. Product inhibition studies were done by studying the reverse reaction. With ADP as product inhibitor competitive inhibition patterns were obtained with AMP and/or MgATP^{2-} as variable substrate. Similar results were obtained for product inhibition by MgADP^- with AMP as variable substrate. The results are consistent with a Rapid Equilibrium Random mechanism. 5. Secondary plots of slope versus product concentration were linear. The data were fitted to the appropriate equation and analysed by computer to give values for the product inhibition constants. 6. Differences between the values of certain kinetic constants for the two forms of the enzyme were observed.

Genetic variation in proteins and enzymes in man appears to fall into two main categories (Harris, 1970). In the first the gene or genes responsible for variant forms of a protein occur only rarely in the population. These so-called 'rare' variants are often associated with some clinical abnormality in the individual concerned because of the altered properties of the variant protein or enzyme. In the second category the gene or genes responsible for alternative form(s) of a particular protein occur with a relatively high frequency ($>1\%$) so that a considerable proportion of the population possesses the common alternative form(s) of that protein.

The alterations in catalytic properties associated with an enzyme variant are not always obvious and sometimes may only be revealed by detailed kinetic studies. For example, Afolayan & Luzzatto (1971) found significant differences in the product-inhibition and substrate-binding properties of the so-called A⁻ variant of erythrocyte glucose 6-phosphate dehydrogenase, although previous work had suggested that the kinetic properties of this variant and the common form of the enzyme were identical (Kirkman *et al.*, 1960; Marks *et al.*, 1961).

It may be argued that when an enzyme variant occurs with a high frequency in the population then

the common and alternative form(s) may be expected to have very similar properties, since otherwise natural selection would favour one or other of the forms (Harris, 1959). This view is supported by the limited evidence available. Scott (1965) measured various kinetic properties of two commonly inherited forms of human erythrocyte acid phosphatase but found no significant difference between them even though the total activity of this enzyme in the erythrocytes of appropriate individuals was markedly different.

We have examined several kinetic parameters of the two common forms of erythrocyte adenylate kinase (Fildes & Harris, 1966) by using initial-velocity and product-inhibition studies in a search for possible catalytic differences. These studies were also designed to see whether the human erythrocyte enzyme has a catalytic mechanism similar to that reported for the rabbit muscle (Rhoads & Lowenstein, 1968) and yeast enzymes (Su & Russell, 1968; Khoo & Russell, 1970).

Experimental

Materials

Enzyme. Erythrocytes used as starting material in these studies were obtained from individual donors whose adenylate kinase type was confirmed on repeat samples of blood. The enzymes were partially purified

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as described in the preceding paper (Brownson & Spencer, 1972) to an approximate specific activity of 20 units/mg of protein. All the kinetic studies reported here were done with enzyme diluted to an appropriate concentration in a solution containing bovine serum albumin (10mg/ml).

Chemicals. The following reagents were obtained from the Boehringer Corporation (London) Ltd., London W.5, U.K.: pyruvate kinase, lactate dehydrogenase, glucose 6-phosphate dehydrogenase, hexokinase, NADP⁺, NADPH, phosphoenol pyruvate, ATP, ADP, AMP. [¹⁴C₈]AMP and [³H₂]ATP were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. and cellulose DE81 paper was from Whatman, Maidstone, Kent, U.K.

Methods

Initial-rate measurements. Initial-rate studies, in which the concentrations of the products were considered negligible, were done with coupled assay systems. The reaction $2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$ is regarded as the forward reaction and the initial rate was measured by using the coupled assay system described by Brownson & Spencer (1972) and based on the method of Oliver (1955). The concentrations of ADP and MgCl₂ were varied as required by the conditions for individual experiments.

A modified version of the coupled system of Bucher & Pfeleiderer (1955) was used to measure initial rates in the reverse direction. Assays were done at 30°C in a total volume of 1 ml. Final concentrations and reagents used in the assay were as follows: tris-HCl, pH 8.0, 100mM; KCl, 120mM; phosphoenolpyruvate, 4mM; NADH, 250μM; lactate dehydrogenase, 0.36 units; pyruvate kinase, 0.15 units. Concentrations of AMP, ATP and MgCl₂ were varied according to the requirements of each experiment. The reaction was started by the addition of enzyme and was measured at 340nm in a Unicam SP.800 spectrophotometer. All measurements were made against cuvettes containing the full reaction mixture except for adenylate kinase. Reaction rate was proportional to the amount of adenylate kinase added provided the extinction change did not exceed 0.03min. Rates measured by the above procedures are expressed as μmol of ADP or ATP produced/min per ml of undiluted enzyme.

Product-inhibition studies. For measurements of initial rates in the presence of products a radiochemical assay method was used. In this procedure it was convenient to study the reverse reaction. In experiments where AMP was the variable substrate, [¹⁴C₈]AMP was used and similarly where ATP was the variable substrate [³H₂]ATP was used. Assays were done in a total volume of 50μl and contained tris-HCl, pH 8, at a final concentration of 100mM; ATP, ADP, AMP and MgCl₂ were added to give the

concentrations required in individual experiments. The assay mixture was preincubated at 30°C for 5min before the addition of 10μl of adenylate kinase to start the reaction. The reaction was terminated by the addition of 10μl of 100mM-EDTA, pH 8.0. Labelled nucleotides in the reaction mixture were separated by ascending chromatography on strips (2.5cm × 25cm) of DEAE (DE81) paper by using the ammonium formate buffer, pH 3.1, described by Ives *et al.* (1963). Samples (50μl) of reaction mixtures were co-chromatographed with unlabelled nucleotides as markers. When MgADP⁻ ('MgADP') was present as product inhibitor concentrations of MgCl₂ up to 50mM were present in the reaction mixtures. Since the additional EDTA necessary to stop the reaction under these conditions interfered with the subsequent chromatographic separation of the labelled nucleotides in these experiments the reaction was terminated by spotting a sample of the reaction mixture directly on to the DEAE-cellulose strip. Chromatograms were irrigated for approx. 4h, dried in the fume cupboard, and the nucleotides detected under a u.v. lamp. Those areas of the strips containing nucleotides were cut out (2.5cm × 5cm) and placed in scintillation vials with 10ml of toluene solution containing 0.5% (w/v) of 2,5-diphenyloxazole and 0.03% (w/v) of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene.

The three nucleotides were well resolved on the chromatograms and only insignificant amounts of radioactivity were found in those areas of the strips between the nucleotide spots. Samples were counted for 5 or 10min in a Packard Tri-Carb model 3001 scintillation spectrometer with an efficiency of 80% for ¹⁴C and 50% for ³H.

The activities of the adenylate kinase preparations were adjusted by dilution so that reaction rates, measured as c.p.m. of radioactive ADP produced/min, were linear with time for at least 10min. Specific radioactivities of the nucleotide substrates were also adjusted by dilution with the appropriate unlabelled nucleotide to give a minimum of 200c.p.m. above background (20c.p.m.) as radioactive ADP, in a 5min reaction period. Each reaction mixture was run in duplicate and rates were corrected by subtracting zero-time blank values. The rate of reaction is expressed as μmol of ADP produced/min per ml of undiluted enzyme. This was calculated from the specific radioactivity of the radioactive substrate determined as follows: the concentration of unlabelled nucleotide used for dilution was determined spectrophotometrically and the radioactivity of diluted nucleotides was determined on portions subjected to the chromatographic procedure described above.

The concentrations of total Mg²⁺ and nucleotide necessary to give the required concentrations of MgATP⁻ ('MgATP') and MgADP for particular

experiments were calculated from the appropriate stability constants of the Mg^{2+} complexes. The values for MgATP and MgADP were taken to be 70000M^{-1} and 2500M^{-1} respectively (Kuby & Noltmann, 1962) and for MgAMP , 100M^{-1} (Nanninga, 1957). From the values of these constants we have assumed that at the concentrations employed Mg^{2+} binds preferentially and completely to ATP in the presence of ADP and also that the formation of MgAMP can be neglected in all experiments where the Mg^{2+} concentration does not exceed the concentration of ATP and/or ADP. The concentrations of stock MgCl_2 solutions were determined by titration against EDTA (Alcock & McIntyre, 1966).

Analysis. The experimental data, in duplicate, were fitted to one of the two following equations (Cleland, 1963c) as appropriate.

Sequential:

$$v = (V[A][B]) / (K_a[B] + K_b[A] + [A][B] + K_{ia}K_b) \quad (1)$$

Linear Competitive Inhibition:

$$v = (V[A]) / \{K(1 + [I]/K_{ia}) + [A]\} \quad (2)$$

where $[A]$, $[B]$ and $[I]$ are the concentrations of substrates and inhibitor respectively, V is the maximum velocity, v the initial velocity and K_a and K_b are Michaelis constants. K_{ia} is an inhibition constant for A and K_{ib} is an inhibition constant obtained from a secondary plot of slope.

The data were analysed and fitted by a Fortran program modified by Dr. D. Peacock, University of Sussex (personal communication) from the one described by Cleland (1963a) using the University of London Atlas computer.

Results and Theory

Initial-velocity analysis

Double-reciprocal plots were derived from initial-velocity measurements in the presence of various concentrations of one substrate at several different constant concentrations of the second substrate. In the forward reaction with either MgADP or ADP as the variable substrate double-reciprocal plots were linear and had a common point of intersection. Within the limits of the experimental data it was not possible to decide whether the point of intersection was on the abscissa or just above it (Figs. 1 and 2). With MgATP as variable substrate in the reverse reaction similar results were obtained (Fig. 3). However, with AMP as variable substrate the point of intersection was slightly below the abscissa (Fig. 4). As this result differed from that obtained with the other three substrates the experiments were repeated with the radiochemical assay with $[^{14}\text{C}_8]\text{AMP}$ as the variable substrate. Reciprocal plots derived from the

results of these experiments again gave a point of intersection below the abscissa.

The families of lines derived with all four substrates are consistent with a sequential mechanism and eliminate the possibility of a Ping Pong mechanism (Cleland, 1963c). Similar results were obtained with both genetic forms of the enzyme.

Fig. 1. Effect of MgADP concentration on the initial velocity of the adenylate kinase reaction measured in the forward direction

The following fixed concentrations of ADP were used: ●, $500\mu\text{M}$; □, $250\mu\text{M}$; ○, $150\mu\text{M}$; ■, $100\mu\text{M}$.

Fig. 2. Effect of ADP concentration on the initial velocity of the adenylate kinase reaction measured in the forward direction

The following fixed concentrations of MgADP were used: ●, $500\mu\text{M}$; □, $250\mu\text{M}$; ○, $150\mu\text{M}$; ■, $100\mu\text{M}$.

The linearity of the secondary plots of vertical intercepts versus reciprocal of concentration of the second substrate were checked graphically before fitting the data to Eqn. 1. Kinetic constants obtained by computer analysis are shown in Table 1.

Product-inhibition analysis

Product-inhibition studies were done to determine which of the three possible sequential mechanisms

Fig. 3. Effect of MgATP concentration on the initial velocity of the adenylate kinase reaction measured in the reverse direction

The following fixed concentrations of AMP were used: ●, 2.22 mM; □, 1.11 mM; ○, 0.56 mM; ■, 0.28 mM.

best describe the action of the enzyme; the mechanisms are Ordered, Theorell Chance or Random Equilibrium (Cleland, 1963b). These studies involved measurements of initial rates of reaction in the presence of various concentrations of one substrate with a fixed non-saturating concentration of the second substrate at several different constant concentrations of one product.

With ADP as a product inhibitor of either AMP or MgATP as variable substrate, plots consistent with linear competitive inhibition were obtained (Figs. 5 and 6). A similar pattern was derived for product inhibition by MgADP with AMP as variable substrate (Fig. 7). In this experiment the high concentrations of Mg^{2+} required to complex all the ADP resulted in the formation of significant amounts of MgAMP. The concentrations of uncomplexed AMP shown in Fig. 7 were calculated from the stability constant of MgAMP (see the Experimental section). The enzyme does not appear to be inhibited by high concentrations of MgAMP as the plots in Fig. 7 are linear; similarly no inhibition was observed in initial-velocity studies with ATP and AMP in the presence of high concentrations of Mg^{2+} (30 mM). In spite of repeated attempts it was not possible to find a suitable concentration of MgADP as product inhibitor that would allow accurate measurements of reaction rates with MgATP as the variable substrate.

The sequential mechanism that fits this product-inhibition data best is Rapid Equilibrium Random (Scheme 1) where the rate-limiting step in the reaction sequence is the interconversion of the ternary

Fig. 4. Effect of AMP concentration on the initial velocity of the adenylate kinase reaction measured in the reverse direction

The following fixed concentrations of MgATP were used: ●, 2.13 mM; □, 1.06 mM; ○, 0.53 mM; ■, 0.27 mM.

Table 1. *Kinetic constants for adenylate kinase types 1 and 2*

Kinetic constants are defined in the text. The constants, $\pm$ S.E.M., were calculated from the appropriate initial-velocity data, from experiments with the linked assay systems.

Substrate	K_m (mM)		K_{ia} (mM)	
	Type 1 enzyme	Type 2 enzyme	Type 1 enzyme	Type 2 enzyme
AMP	0.33 ± 0.10	0.27 ± 0.03	0.18 ± 0.06	0.42 ± 0.10
AMP*	0.59 ± 0.29		0.28 ± 0.11	
MgATP	0.95 ± 0.13	0.40 ± 0.05	1.54 ± 0.56	0.77 ± 0.11
MgATP*	1.19 ± 0.45			
ADP	0.12 ± 0.03	0.08 ± 0.01	0.24 ± 0.06	0.15 ± 0.03
MgADP	0.22 ± 0.04	0.20 ± 0.04	0.19 ± 0.04	0.96 ± 0.28

* Values obtained from initial-velocity studies with the radiochemical assay.

Fig. 5. Product inhibition of the adenylate kinase reaction measured in the reverse direction with AMP as variable substrate

The concentration of MgATP was fixed at 5 mM ($[ATP] = [MgCl_2] = 5 \text{ mM} = [MgATP]$). Product inhibitor, ADP, was tested at the following concentrations: ●, no ADP; □, 1.5 mM; ○, 3.7 mM; ■, 7.4 mM.

complex. The complete rate equation for this mechanism is (Cleland, 1963b):

$$v = \frac{V_1 V_2 \left([A][B] - \frac{[P][Q] V_1 K_p K_{iq}}{V_2 K_{ia} K_b} \right)}{K_b [A] V_2 + K_a [B] V_2 + A[B] V_2 + K_{ia} K_b V_2 + \frac{K_q [P] K_{ia} K_b V_2}{K_p K_{iq}} + \frac{[Q] K_{ia} K_b V_2}{K_{iq}} + \frac{[P][Q] K_{ia} K_b V_2}{K_{iq} K_p}} \quad (3)$$

where K_p is a Michaelis constant and K_{iq} is an inhibition constant for Q.

Scheme 1. *Rapid Equilibrium Random reaction mechanism*

The rate equation for this mechanism is given in eqn. (3), and the symbols are as follows: E, enzyme; A and B, substrates; P and Q, products.

By setting the concentration of one product equal to zero, Eqn. 3 modifies to the form shown in eqn. 4:

$$\frac{1}{v} = \frac{K_b}{V} \left(1 + \frac{K_{ia}}{[A]} \left(1 + \frac{[Q]}{K_{iq}} \right) \right) \frac{1}{[B]} + \frac{1}{V} \left(1 + \frac{K_a}{[A]} \right) \quad (4)$$

This equation may be rearranged, depending on which substrate is being varied and which product is acting as inhibitor. By inspection it is apparent that changes in the concentration of the product inhibitor (Q) will affect only the slope of double-reciprocal plots and not the intercept. Inhibition by each product against either substrate yields an analogous equation which describes competitive inhibition.

Secondary plots of slope against product concentration were checked graphically for linearity and

then fitted to eqn. (2) by using the computer. The values of K_{is} thus obtained were used to calculate values for K_{iq} by using the following relationship, which was derived from eqn. (4):

$$K_{is} = K_{iq} \left(1 + \frac{[A]}{K_{ia}} \right)$$

These values are given in Table 2.

Discussion

Initial-velocity studies together with product-inhibition studies reported here are in accord with a Rapid Equilibrium Random mechanism for erythrocyte adenylate kinase. Yeast and rabbit muscle enzymes also appear to act via a random mechanism although in the latter case the rate-limiting step in the

reaction sequence is probably at some stage other than the interconversion of the ternary complex. The kinetic parameters listed in the present work are of similar magnitude to those given by Khoo & Russell (1970) for the yeast enzyme. With both the yeast and the muscle enzyme evidence has been produced that AMP acts as an inhibitor under certain conditions. In the case of the yeast enzyme with MgATP as the fixed substrate, high concentrations of the variable substrate (AMP) were inhibitory (Khoo & Russell, 1970). Isotope equilibrium experiments of

Fig. 6. Product inhibition of the adenylate kinase reaction measured in the reverse direction

The concentration of AMP was fixed at 5 mM. The various concentrations of MgATP were obtained by using equimolar concentrations of ATP and MgCl₂. Product inhibitor, ADP, was tested at the following concentrations: ●, no ADP; □, 0.292 mM; ○, 0.585 mM; ■, 1.17 mM.

Fig. 7. Product inhibition of the adenylate kinase reaction measured in the reverse direction

The concentration of MgATP was fixed at 5 mM. Product inhibitor, MgADP, was tested at the following concentrations: □, 0.31 mM; ○, 1.6 mM; ■, 3.1 mM. All reaction mixtures contained 50 mM-MgCl₂ to fully complex ATP and ADP. The concentrations of the variable substrate, AMP, were calculated by using the stability constant for MgAMP (see the Experimental section).

Table 2. Product inhibitor constants for adenylate kinase types 1 and 2

The constants are defined in the text; values are given $\pm$ S.E.M. and are based on data obtained with the radio-chemical assay.

Fixed substrate	Variable substrate	Product inhibitor	K_i (mM)	
			Type 1 enzyme	Type 2 enzyme
MgATP	AMP	ADP	0.062 ± 0.022	0.11 ± 0.03
MgATP	AMP	MgADP	0.044 ± 0.015	0.093 ± 0.029
AMP	MgATP	ADP	0.053 ± 0.020	0.025 ± 0.006

Rhoads & Lowenstein (1968) on the muscle enzyme also gave evidence of substrate inhibition by AMP and it was suggested that the actual inhibitor was MgAMP. In the experiments described here no such inhibition was observed even when the MgAMP concentration was high.

In initial-velocity studies with adenylate kinase types 1 and 2 the patterns of intersecting lines with MgATP as fixed substrate (Fig. 4) indicate that binding of this nucleotide hinders the binding of the variable substrate (AMP) (Westley, 1969). This contrasts with the results obtained with yeast and muscle adenylate kinase where binding of MgATP facilitates binding of AMP. However, AMP as fixed substrate facilitates the binding of MgATP to adenylate kinase from all three sources. Results with the erythrocyte enzymes can be interpreted in terms of Koshland's induced-fit mechanism of enzyme action (Koshland, 1958). Presumably the binding of MgATP induces a conformational change in the enzyme which hinders subsequent binding of AMP whilst the conformation induced by first binding AMP facilitates binding of MgATP.

The validity of the proposed reaction mechanism for the erythrocyte enzymes could not be tested by application of the appropriate Haldane relationships (Haldane, 1930) as the V values obtained were not comparable from one experiment to another because of instability of the enzyme. It was not possible to obtain all four product-inhibition patterns for the reverse reaction as noted previously. For these reasons it would be desirable to further investigate the mechanism by isotope exchange studies.

A limited number of kinetic studies have been done on those erythrocyte enzymes that are known to exist in common alternative forms. The results suggest that any kinetic differences which exist between the various forms of an enzyme are extremely small. Scott (1965) working with acid phosphatase partially purified from erythrocytes of individuals of phenotypes AA and BB (Hopkinson *et al.*, 1963) found no significant differences in K_m and V at various pH values. The relative activities of the two enzymes with a variety of substrates were essentially identical. No differences were found between the two enzymes regarding their inhibitor constants for inhibition by inorganic phosphate; Fenton & Richardson (1971) confirmed these findings. Another erythrocyte enzyme that exists in common alternative forms, namely glucose 6-phosphate dehydrogenase, has also been studied in some detail. Binding constants for both NADP⁺ and NADPH were similar (Afolayan & Luzzatto, 1971) for forms A and B (World Health Organization, 1967). The present results (Tables 1 and 2) suggest

that differences do exist between certain kinetic parameters for the two common forms of erythrocyte adenylate kinase. These differences are not sufficiently clear-cut to warrant a discussion of their possible physiological significance but suggest that further detailed studies may be informative.

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