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Some Properties of Human Skeletal Muscle Creatine Kinase

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Abstract

Creatine kinase has been purified from human skeletal muscle. The properties of the human enzyme are similar to those of the enzyme from rabbit muscle. The molecular weight was determined as approximately 80 000 with a probable two reactive sulphydryl groups per molecule. Manganous (II) ion was almost as effective as magnesium as the activating metal ion, and calcium and cobalt could also act in this capacity. Under standardized conditions the nucleotide specificity was ADP > dADP > IDP > GDP > UDP > XDP in the reverse reaction. No hydrolytic activity was observed with ATP.

Initial velocity and product inhibition studies were used to determine various kinetic constants for the substrates of the enzyme. It was concluded that, as for the rabbit muscle enzyme, the reaction probably followed a rapid equilibrium random mechanism. Anomalous kinetic behaviour, however, was observed for the forward reaction for $MgATP^{2-}$ but not for creatine, when measurements were extended over a much wider range than normally used. The reciprocal plot of velocity as a function of substrate concentration gave a curve, concave downwards, instead of a straight line.

Introduction

Creatine kinase (ATP: creatine *N*-phosphotransferase; EC 2.7.3.2) occurs in high concentrations in mammalian muscle. The enzyme was first isolated from rabbit muscle (Kuby *et al.* 1954) though it has been subsequently purified from a number of species (Watts 1973). The rabbit muscle enzyme, in particular, has undergone intensive investigation as the prototype of kinase-type reactions (Kuby and Noltmann 1962; Morrison and James 1965; Cohn 1970).

We have recently had occasion to purify creatine kinase from human skeletal muscle as a prerequisite for the establishment of a radioimmunoassay for creatine kinase in serum (Nicholson and O'Sullivan 1975). We present here a description of some properties of the enzyme. The study complements and extends the preliminary work of Kumuduvalli *et al.* (1970) and the physicochemical studies of Keutel *et al.* (1972).

Materials and Methods

Materials

Phosphorylcreatine, α-naphthol (grade III recrystallized), DEAE-cellulose, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid), blue dextran, bovine chymotrypsin, dithiobisnitrobenzoic acid (DTNB), and the disodium salts of ATP, ADP, dADP, IDP, UDP, and XDP were all obtained from Sigma Chemical Co. *N*-Ethylmorpholine, obtained from Eastman–Kodak, was purified by redistillation, and adjusted to pH 8·0 with 5 M HCl. DEAE-Sephadex was obtained from Pharmacia. Ammonium sulphate was an ultra pure, special enzyme grade from Schwarz Bio-

research, New York. The disodium salt of EDTA was from British Drug Houses and 2-mercaptoethanol was purchased from Merck.

Ethanol (95%) and the chloride salts of Mg^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+} and $ZnSO_4$ were all analytical grade and were obtained from Ajax Chemicals Ltd. Human haemoglobin was from Calbiochem, U.S.A. Rat 7 S γ-globulin was obtained from Miles Pentex Ltd and 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (DCTA) from Hopkin and Williams Ltd, England. [^{14}C]ATP (Lot No. 570550; 0·02 μmol; $1{\cdot}44\times10^7$ dpm) was obtained from International Chemical and Nuclear Corp. and was diluted 1/100 in 10 mM ATP. [^{14}C]ADP, specific activity 42 μCi/mmol (Lot No. 6603; 0·05 μmol; 50000 dpm) was obtained from Schwarz Bioresearch Inc. This was diluted 1/20 in 10 mM ADP stock solution. All other chemicals used were A.R. grade.

Enzyme Assay

Creatine kinase activity in the reverse direction was followed by the α-naphthol diacetyl reaction for the determination of creatine as described by Morrison *et al.* (1961). For routine assays, the reaction mixture contained 0·1 M *N*-ethylmorpholine–HCl (pH 8·0), 2 mM $MgCl_2$, 0·01 mM EDTA, 1 mM ADP and 10 mM phosphorylcreatine in a total volume of 1·0 ml.

The α-naphthol method could not be used for product inhibition studies with creatine as the product under examination. In this case the reaction was followed by the use of [^{14}C]ADP. Incubation mixtures contained 0·05 M HEPES–KOH (pH 8·0), 10 μM EDTA, plus appropriate concentrations of $MgCl_2$, [^{14}C]ADP (5000 dpm diluted with 10 mM ADP), phosphorylcreatine and creatine in a total volume of 200 μl. Control tubes contained no enzyme. The reaction was commenced by addition of 0·3 μg enzyme. After incubation (usually 2 min) at 30°C the reaction was stopped by application of 10-μl samples of the reaction mixture on the No. 1 Whatman chromatography paper (25 by 40 cm) followed by rapid drying in a cold air draught. Nucleotide markers (ATP, ADP, and AMP) were added to the chromatogram to facilitate location of the nucleotide spots. The paper was developed and the spots located and counted as described elsewhere (Lee and O'Sullivan 1975).

A similar procedure was used for the studies in the forward direction, over a wide concentration of $MgATP^{2-}$ (see Fig. 6), except that much higher specific activity of [^{14}C]ATP (53 mCi/mmol) was used in order to increase the sensitivity at low substrate concentration.

Hydrolytic Activity of Creatine Kinase

A similar procedure, using [^{14}C]ATP, was used to test the activity of the enzyme as a possible ATPase. The reaction mixture contained 2 mM [^{14}C]ATP ($1{\cdot}5\times10^4$ dpm) and 3 mM $MgCl_2$ diluted in 50 μl 20 mM dithiothreitol, and 40 μl 0·5 M HEPES, pH 8·0, in a total volume of 400 μl. The reaction was started by the addition of 10 μg creatine kinase and incubated at 30°C for time periods up to 1 h. The reaction was stopped by application of 10-μl samples on to No. 1 Whatman chromatography paper. Separation of the nucleotides and counting techniques were the same as for the isotopic-exchange experiments.

Protein Estimation

Protein concentrations in crude fractions containing the enzyme were estimated by the method of Warburg and Christian (1941). The protein concentration of relatively pure enzyme was estimated from the specific absorption of 0·888 mg ml^{-1} cm^{-1} (Kuby and Noltmann 1962).

Electrophoretic Techniques

Polyacrylamide disc electrophoresis was carried out in 7½% polyacrylamide gel, using the system described by Davis (1964) with tris–glycine and tris–HCl buffers, pH 8·3, as upper and lower buffers respectively. Electrophoresis was carried out at 4°C, 4 mA per tube, until a bromphenol blue marker reached the bottom of the gel. Gels were stained with Coomassie brilliant blue and excess stain in the gels removed by washing in 7% acetic acid.

Double Immunodiffusion

Double immunodiffusion was carried out using the method described by Ouchterlony (1967). A 1·5% agar solution, pH 7·0, was prepared by dissolving 15 g of agar in 1 litre of a solution made up of 500 ml of 0·165 M NaCl, 90 ml 0·1 M citric acid, and 410 ml 0·1 M Na_2HPO_4. Sodium azide, 1 g/l, was added as preservative. Antibody to purified enzyme was prepared as described by Nicholson and O'Sullivan (1973).

Molecular Weight Estimation by Gel-filtration Chromatography

Sephadex G150 (Pharmacia) was calibrated by determination of the void volume using blue dextran and individual determinations of the elution volumes of proteins of known molecular weight, namely haemoglobin (68 000), rabbit muscle creatine kinase (82 600), chymotrypsin (21 600) and rat 7 S γ-globulin (160 000). Elution volumes were obtained by monitoring the eluate at 280 nm, or at 405 nm in the case of haemoglobin, and plotted against the logarithm of their molecular weight according to the method of Andrews (1964).

Preparation of Muscle Homogenate

Cadaver quadriceps muscle was removed less than 7 h after death. The muscle was homogenized in a Sorvall blender with an equal volume of 0·01 M KCl containing 0·1 mM EDTA and 1 mM mercaptoethanol. The homogenate was centrifuged at 10 000 g and sodium azide added to the supernatant to a concentration of 0·0002% (w/v).

Enzyme Purification

When this work was initiated, a satisfactory procedure for the purification of human muscle creatine kinase had not been described, except for the starch block electrophoresis method of Kumuduvalli *et al.* (1970), which yielded small recoveries of low activity enzyme. Three procedures were explored: (1) adaptation of the ethanol fractionation method of Kuby *et al.* (1954) for the rabbit muscle enzyme; (2) acetone fractionation followed by chromatography on DEAE-cellulose and Sephadex G150; and (3) adaptation of a method principally using ammonium sulphate precipitation, developed by D. W. Watts (personal communication) for the preparation of creatine kinase from monkey muscle. An outline of the first of these procedures is given below. Further details can be obtained from the authors. During the course of the work, the method of Keutel *et al.* (1972) was published and this procedure was also used.

Alcohol Fractionation

The human muscle enzyme appeared to behave in a similar fashion to that from rabbit muscle except for the final fractionation step. The procedure involved precipitation of protein from the crude homogenate at pH 9·0 with 1·5 volumes of 95% (v/v) ethanol. Following centrifugation, 1 M $MgSO_4$ was added to 0·03 M, and the precipitate collected and extracted with 0·07 M magnesium acetate (6 and 4%, respectively, of the initial volume). It was found that the enzyme started to precipitate at lower concentrations of ethanol than was observed for the rabbit muscle enzyme. Protein precipitating between 36 and 50% of 95% (v/v) ethanol was collected, but was found to still contain a number of minor impurities. These were removed by successive chromatography on phosphocellulose and DEAE-Sephadex.

Analysis of Kinetic Data

The data were analysed with computer programs described by Cleland (1963). The linearity of all plots was initially checked graphically and the kinetic constants were obtained by analysis of the primary plots with SEQUEN, NONCOMP and COMP computer programs.

Results

Enzyme Purification

A number of procedures yielded substantial purification of creatine kinase. An outline of the ethanol fractionation is given in Table 1. The preparation showed only a single band on polyacrylamide gel electrophoresis (Fig. 1*a*). The purity was confirmed by the observation of a single immunoprecipitin line against antiserum to the pure enzyme. There was also a single precipitation line against extracts from human skeletal muscle and heart muscle but no cross-reactivity with extracts from brain and cerebellum (Fig. 1*b*). The various procedures yielded enzyme of lower specific activity than that of Keutel *et al.* (1972), namely 108 μmol creatine formed per milligram protein per minute from the ethanol fractionation, 85 μmol from acetone fractionation (followed by DEAE chromatography) and 91 μmol by the ammonium

sulphate fractionation, compared to 125 μmol reported by Keutel *et al.* However, the procedures were considerably faster and did yield enzyme of sufficient purity for many purposes, for example the raising of antibodies (Nicholson and O'Sullivan 1973). The specific activities were three- to fourfold higher than obtained by Kumuduvalli *et al.* (1970).

Table 1. Preparation of human creatine kinase by alcohol fractionation and gel chromatography

Fraction	Volume (ml)	Protein concn (mg/ml)	Total protein (mg)	$10^{-4}\times$ Total activity[A]	Specific activity[B]	Purification	Recovery of activity (%)
Homogenate (in 0·01 M KCl)	1140	11·6	13000	17·1	13		100
(*a*) 1·5 vols 95% ethanol	2530	1·7	4300	14·2	33	2·5	83
(*b*) $MgSO_4$ precipitation, Mg acetate extraction	124	8·9	110	5·4	49	3·8	32
(*c*) Alcohol fractionation and dialysis	41	6·6	270	1·7	62	4·8	10
Phosphocellulose chromatography	62	2·6	160	1·3	83	6·5	8
DEAE-Sephadex chromatogrpahy	180	0·4	76	0·8	108	8·3	5

[A] Measured in enzyme units.
[B] Measured as micromoles creatine per milligram protein per minute.

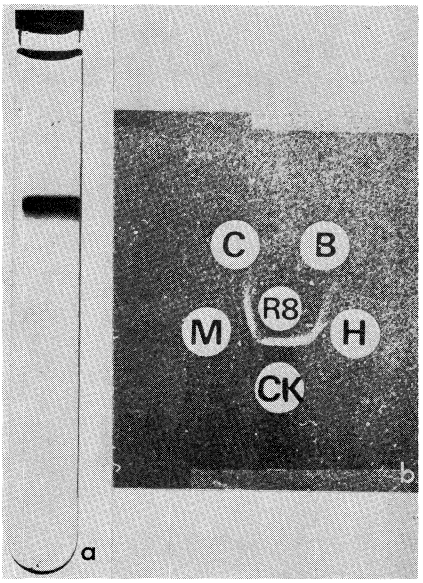

Fig. 1. (*a*) Polyacrylamide disc electrophoresis of human muscle creatine kinase purified by ethanol fractionation followed by chromatography on phosphocellulose and DEAE-Sephadex (Table 1). (*b*) Double immunodiffusion of creatine kinase antiserum against pure creatine kinase and various tissue extracts. The centre well (*R8*) contained rabbit antiserum raised against pure enzyme. The peripheral wells contained pure muscle creatine kinase (*CK*) and homogenates from human skeletal muscle (*M*), cerebellum (*C*), brain (*B*) and heart muscle (*H*).

Metal Ion Specificity

The effect of divalent cations on the activity of human muscle creatine kinase in 0·05 M HEPES–KOH, pH 8·0, was examined at a metal-ADP concentration of 1·0 mM and a phosphorylcreatine concentration of 10 mM using previously reported values for the metal stability constants (O'Sullivan and Perrin 1964; Phillips 1966). The relative order under these conditions was $MgADP^- > MnADP^- > CaADP^- > CoADP^-$ (100 : 86 : 44 : 30). No activity was observed with $ZnADP^-$ or $SrADP^-$.

Substrate Specificity

Under the conditions tested, with 1 mM Mg-nucleotide and 10 mM phosphorylcreatine, the ability of nucleoside diphosphates to act as substrates decreased in the order $MgADP^- > MgdADP^- > MgIDP^- > MgGDP^- > MgUDP^- > MgXDP^-$ (100 : 27 : 9 : 2 : 1 : 1).

Sulphydryl Groups

Estimation of –SH groups with DTNB (Ellman 1959) gave values from 1·2 to 2·0 with different preparations of enzyme, with specific activities ranging from 85 to 108 units.

Molecular Weight Determination

The approximate molecular weight obtained from a plot of elution volume against the logarithm of the molecular weight of standard proteins (see Materials and Methods) was 80 000 (Fig. 2).

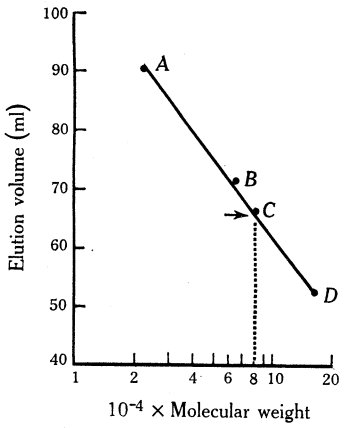

Fig. 2. Molecular weight determination of human creatine kinase on a Sephadex G150 column. The elution volumes of four reference proteins are plotted against their molecular weights: *A*, chymotrypsin; *B*, haemoglobin; *C*, rabbit creatine kinase; *D*, rat 7 S γ-globulin. The elution volume of human muscle creatine kinase is indicated by the arrow.

Initial Velocity Studies

Plots of initial velocity at varying $MgADP^-$ concentrations with various fixed concentrations of phosphorylcreatine are shown in Fig. 3*a*; and at varying phosphorylcreatine concentrations with various fixed concentrations of $MgADP^-$ in Fig. 3*b*. Both series of plots converged on the abscissa indicating that the apparent Michaelis constant for one substrate was not substantially affected by the concentration of the other. Kinetic constants were determined by computer analysis of the data for Fig. 3 using the SEQUEN computer program of Cleland (1963). They were $0·26 \pm 0·11$ and $0·23 \pm 0·06$ mM for K_{ia} and K_a, the dissociation constants of MgADP from MgADP–enzyme and MgADP–enzyme–phosphorylcreatine respectively; and $3·96 \pm 1·69$ and $3·47 \pm 0·91$ mM for K_{ib} and K_b, the dissociation constants of phosphorylcreatine–enzyme, and phosphorylcreatine–enzyme–MgADP respectively.

Product Inhibition Studies

Results from product inhibition studies of the reverse reaction are illustrated in Figs 4 and 5, namely $MgATP^{2-}$ with respect to $MgADP^-$ (Fig. 4*a*); $MgATP^{2-}$ with

respect to phosphorylcreatine (Fig. 4*b*); creatine with respect to $MgADP^-$ (Fig. 5*a*); and creatine with respect to phosphorylcreatine (Fig. 5*b*). The pattern of inhibition and the calculated inhibition constants are collected in Table 2.

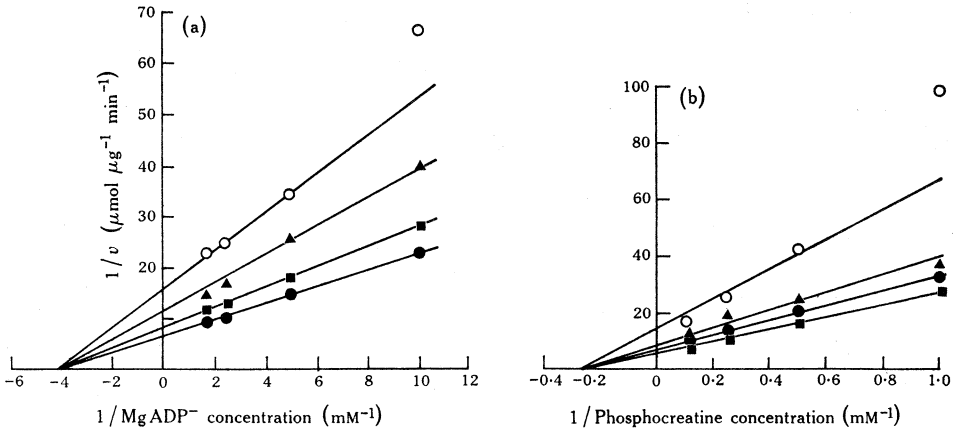

Fig. 3. (*a*) Initial velocity pattern for the reverse reaction with $MgADP^-$ as the variable substrate at different fixed concentrations of phosphorylcreatine: ○ 1·5 mM; ▲ 3 mM; ■ 6 mM; ● 10 mM. (*b*) Initial velocity pattern for the reverse reaction with phosphorylcreatine as the variable substrate at different fixed concentrations of $MgADP^-$: ○ 0·15 mM; ▲ 0·3 mM; ● 0·6 mM; ■ 1·0 mM. Velocities are expressed as micromoles creatine per microgram creatine kinase per minute.

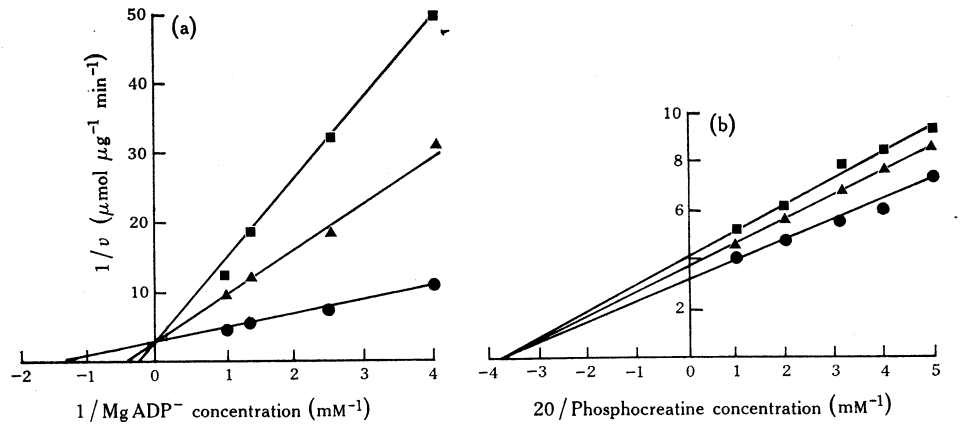

Fig. 4. Product inhibition of the reverse reaction of $MgATP^{2-}$ with (*a*) $MgADP^-$ as the variable substrate and phosphorylcreatine held constant at 5·0 mM, and (*b*) with phosphorylcreatine as the variable substrate and $MgADP^-$ held constant at 0·4 mM. The concentrations of $MgATP^{2-}$ were: ● 0; ▲ 0·2 mM; ■ 0·4 mM. Velocities are expressed as micromoles creatine per microgram creatine kinase per minute.

Hydrolytic Activity of Human Creatine Kinase

No significant breakdown of ATP was observed with incubation periods of up to 1 h under the conditions tested.

Initial Velocities over a Wide Range of Substrate Concentration

While this work was being carried out our attention was drawn to some recent work of R. K. Scopes (unpublished data) on the reaction catalysed by yeast phosphoglycerate kinase. Scopes found that conditions of substrate concentration could be found under which the enzyme appeared to obey the requirements for a simple sequential mechanism. However, by going to very low (10·0 μM) and very high (5·0 mM) concentrations of $MgATP^{2-}$, quite different apparent Michaelis constants could be obtained. The overall effect was that a Lineweaver–Burke plot could be portrayed as a curve concave downwards.

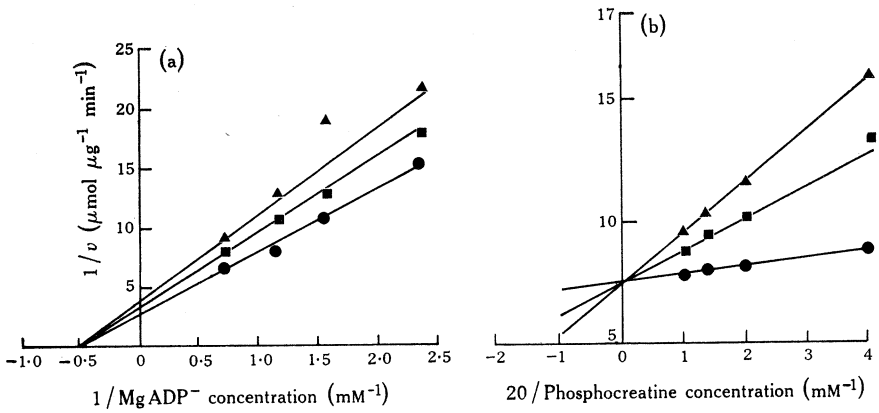

Fig. 5. Product inhibition of the reverse reaction by creatine (*a*) with $MgADP^-$ as the variable substrate and phosphorylcreatine held constant at 10 mM, and (*b*) with phosphorylcreatine as the variable substrate and $MgADP^-$ held constant at 1·0 mM. The concentrations of creatine were: ● 0; ■ 10 mM; ▲ 20 mM. Velocities are expressed as micromoles $MgATP^{2-}$ per microgram creatine kinase per minute.

Table 2. Inhibition constants for the creatine kinase reaction

Constants for product inhibition of the reverse reaction catalysed by human creatine kinase were obtained from the computer analysis of results for the data in Figs 4 and 5. Values for K_i andd K_I were calculated using equations for the relationship of apparent and real inhibition constants given by Morrison and James (1965). Results in parentheses are values reported by these authors for the rabbit muscle enzyme

Product inhibitor	Variable substrate	Type of inhibition[A]	Apparent inhibition constants		True inhibition constants		Concn of constant substrate (mM)	
			K_{is} (mM)	K_{ii} (mM)	K_i (mM)	K_I (mM)		
Creatine	$MgADP^-$	n.c.	45±23	49±28 (44±2)	12·9 (13)	12·5 (9·8)	PC	10·0
Creatine	PC[B]	c.	3·3±0·2		0·98		$MgADP^-$	1·00
$MgATP^{2-}$	$MgADP^-$	c.	0·67±0·05		0·59 (0·68)		PC	5·0
$MgATP^{2-}$	PC	n.c.	13·0±5·4 (3·91±0·4)	12·2±3·0 (20±3)	5·1 (1·16)	4·5 (2·26)	$MgADP^-$	0·4

[A] n.c., non-competitive; c, competitive.
[B] Phosphorylcreatine.

It was considered to be of interest to see if human creatine kinase could be demonstrated to behave in a similar fashion. Results of such a study, with $MgATP^{2-}$ as the

variable substrate over the range, are shown in Fig. 6. Deviations from Michaelis–Menten behaviour, towards higher velocities, were observed with increasing concentrations of $MgATP^{2-}$ as substrate. Thus, the reciprocal plot of $1/v$ against $1/MgATP^{2-}$ gave a curve, concave downward, instead of a straight line. The lines drawn in Fig. 6 correspond to K_m values of 0·22 mM (full line), 0·8 mM (dotted line) and 8·9 mM (inset).

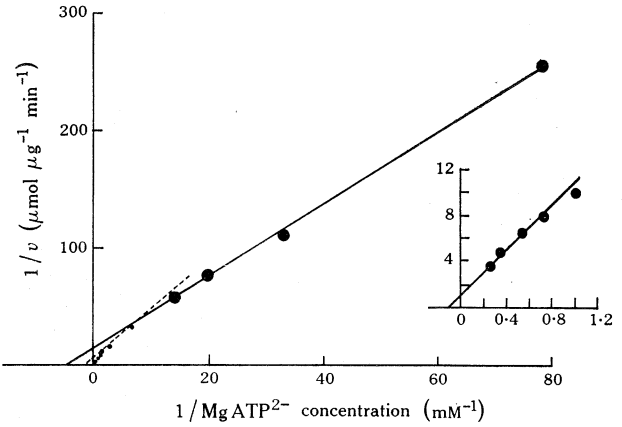

Fig. 6. Double reciprocal plot of velocity with respect to $MgATP^{2-}$ concentration. Assays were carried out in 50 mM HEPES–KOH, pH 8·0, with 40 mM creatine, at 30°C. Other details are given in the text.

A similar experiment was carried out with creatine as the variable substrate (0·5–20·0 mM) with $MgATP^{2-}$ kept constant at 1·5 mM. In this case the plot was linear from 0·5 to 10·0 mM. Above 10·0 mM creatine, substrate inhibition was observed; the reciprocal plot was concave upwards. Similar experiments in the reverse direction with $MgADP^{-}$ varied from 0·01 mM to 10·0 mM and phosphorylcreatine at 10·0 mM did not produce any evidence of curvature. An experiment carried out under identical conditions as that shown in Fig. 6 but with rabbit muscle enzyme instead of human muscle enzyme, produced essentially similar results.

Discussion

The properties of the human muscle isoenzyme of creatine kinase reported in this paper are similar to those obtained for the rabbit muscle enzyme with respect to substrate and metal ion specificity (Watts 1973). Manganous, calcium and cobalt ions were all effective in the latter capacity and the enzyme could use dADP and IDP as moderate substrates, and UDP and XDP as poor substrates.

The kinetic results for the human enzyme in the reverse direction are also in substantial agreement with those for the rabbit enzyme using comparable conditions (Morrison and James 1965). The initial velocity plots indicated a sequential mechanism and the pattern of product inhibition would be compatible with either a rapid equilibrium random mechanism or a Theorell–Chance mechanism. The former, as for the rabbit muscle enzyme, would appear the more likely.

Though the definitive experiments to distinguish between these two mechanisms were not carried out, it was suggested by a reviewer that it is possible to test the fit

of a Theorell–Chance mechanism by secondary plots of the product inhibition data. Thus, secondary plots of the slopes and vertical intercepts of the data from Figs 4 and 5 were carried out. From Fig. 4, the K_i (slope) for $MgATP^{2-}$ with respect to $MgADP^-$ was 0·08 mM, whereas K_i (slope) and K_i (intercept) for $MgATP^{2-}$ with respect to phosphorylcreatine were 0·40 and 0·49 mM respectively. Similarly, data from Fig. 5 gave K_i (slope) for creatine with respect to phosphorylcreatine as 4·2 mM, and K_i (slope) and K_i (intercept) as 13·3 and 10·6 mM, respectively, for creatine with respect to $MgATP^{2-}$. These calculations would indicate a rapid random equilibrium mechanism as the more likely, because for a Theorell–Chance mechanism the apparent inhibitor constants would not vary with the concentration of the fixed substrate.

The small degree of synergism between the binding of $MgADP^-$ and phosphorylcreatine ($K_a < K_{ia}$ by a factor of approximately 3) to the rabbit muscle enzyme (Morrison and James 1965) was not observed for the human muscle enzyme ($K_a \sim K_{ia}$; $K_b \sim K_{ib}$). This would reflect a small difference in the binding of substrates to the two enzymes; though any synergism of much smaller magnitude would be considered to be within the experimental error of the study.

The results for the forward direction illustrated in Fig. 6, which were reproducible with both human and rabbit muscle, are intriguing and we are not able to offer a satisfactory explanation for them. As mentioned above, the reverse reaction catalysed by the rabbit muscle enzyme has been analysed in considerable detail and shown to follow a rapid equilibrium random mechanism (Morrison and James 1965). It is possible that what is being observed in our results is a relatively minor kinetic pathway which only becomes relevant under certain conditions of substrate concentration. It could indicate pitfalls in the determination of K_m values over a restricted range of substrate concentration. The results are strikingly similar to those obtained by Scopes (unpublished data) for yeast phophoglycerate kinase. We might note in particular that for both enzymes the anomalous kinetics are seen only in the forward direction, not the reverse direction (cf. Lee and O'Sullivan 1975).

The data could be interpreted in terms of negative cooperativity (with respect to binding constants) or substrate activation (with respect to initial velocity). It has long been assumed that the two subunits of creatine kinase are identical and that the two active sites per molecule of dimer act independently of each other (Kuby and Noltmann 1962; Watts 1973). Two recent publications have questioned this assumption. McLaughlin (1974), using a fluorescent probe technique, reported that the binding of ADP to the rabbit muscle enzyme in the presence of creatine plus nitrate ion displayed features similar to negative cooperativity. Subsequently, Price and Hunter (1976) from studies of the two reactive sulphydryl groups of the enzyme also obtained evidence for the binding of ADP, again in the presence of creatine and nitrate ion, as indicative of negative interactions between the subunits. [From magnetic resonance studies it appears that the combination of metal-ADP^-, creatine and nitrate ion simulates the active species of the working enzyme, the nitrate ion occupying the site normally taken up by the phosphoryl group being transferred in the reaction (Reed and Cohn 1972).] Hill plots (Hill 1910) from various experiments, however, were all in the range 0·90–1·00, which would argue against a significant degree of cooperativity.

Finally, we could note that Ali and Brownstone (1976) have published kinetic evidence for negative cooperativity with red cell phosphoglycerate kinase, a monomeric enzyme. It is diffcult to assess their results as they were related to total magnesium and to total substrate (ADP and ATP, and 1,3-diphosphoglycerate respectively), and

no attempt was made to calculate the concentration of the metal–substrate species relevant to the reaction.

Acknowledgments

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References

Ali, M., and Brownstone, Y. S. (1976). A study of phosphoglycerate kinase in human erythrocytes. *Biochim. Biophys. Acta* **445**, 89–103.

Andrews, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* **91**, 222–33.

Cleland, W. W. (1963). Computer programmes for processing enzyme kinetic data. *Nature (London)* **198**, 463–5.

Cohn, M. (1970). Magnetic resonance studies of enzyme–substrate complexes with paramagnetic probes as illustrated by creatine kinase. *Q. Rev. Biophys.* **3**, 61–89.

Davis, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404–27.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70–7.

Hill, A. V. (1910). The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J. Physiol. (London)* **40**, Proceedings, iv-vii.

Keutel, H. J., Okabe, K., Jacobs, H. K., Ziter, F., Maland, L., and Kuby, S. A. (1972). Studies on adenosine triphosphate transphorylases. XI. Isolation of the crystalline adenosine triphosphate-creatine transphosphorylases from the muscle and brain of man, calf and rabbit; and a preparation of their enzymatically active hybrids. *Arch. Biochem. Biophys.* **150**, 648–78.

Kuby, S. A., Noda, L., and Lardy, H. A. (1954). Adenosinetriphosphate-creatine transphosphorylase. I. Isolation of the crystalline enzyme from rabbit muscle. *J. Biol. Chem.* **209**, 191–201.

Kuby, S. A., and Noltmann, E. A., (1962). ATP–creatine transphosphorylase. *The Enzymes* **6**, 515–603.

Kumudavalli, I., Moreland, B. H., and Watts, D. C. (1970). Properties and reaction with iodoacetamide of adenosine 5′-triphosphate-creatine phosphotransferase from human skeletal muscle. *Biochem. J.* **117**, 512–23.

Lee, C. S., and O'Sullivan, W. J. (1975). Properties and mechanism of action of human erythrocyte phosphoglycerate kinase. *J. Biol. Chem.* **250**, 1275–81.

McLaughlin, A. C. (1974). The interaction of 8-anilino-1-naphthalenesulfonate with creatine kinase. *J. Biol. Chem.* **249**, 1445–52.

Morrison, J. F., and James, E. (1965). The mechanism of the reaction catalysed by adenosine triphosphate creatine phosphotransferase. *Biochem. J.* **97**, 37–52.

Morrison, J. F., O Sullivan, W. J., and Ogston, A. G. (1961). Kinetic studies of the activation of creatine-phosphoryltransferase by magnesium. *Biochim. Biophys. Acta* **52**, 82–96.

Nicholson, G. A., and O'Sullivan, W. J. (1973). A radioimmunoassay for creatine kinase. *Proc. Aust. Assoc. Neurol.*, 105–8.

Nicholson, G. A., and O'Sullivan, W. J. (1975). Radioimmunoassay for human creatine kinase: the role of enzyme inactivation in serum creatine kinase estimations. 'Recent Advances in Myology'. [Excerpta Medica International Congress Series No. 360, 267–272.]

O'Sullivan, W. J., and Perrin, D. D. (1964). The stability constants of metal–adenine nucleotide complexes. *Biochemistry* **3**, 18–26.

Phillips, R. C. (1966). Adenosine nucleotides ionisation, metal complex formation and conformation in solution. *Chem. Rev.* **66**, 501–27.

Ouchterlony, Ö. (1967). Immunodiffusion and immunoelectrophoresis. In 'Handbook of Experimental Immunology'. (Ed. D. M. Weir.) pp. 655–706. (Blackwell: Oxford.)

Price, N. C., and Hunter, M. G. (1976). Non-identical behaviour of the subunits of rabbit muscle creatine kinase. *Biochim. Biophys. Acta* **445**, 364–76.

Reed, G. H., and Cohn, M. (1972). Structural changes induced by substrates and anions at the active site of creatine kinase. *J. Biol. Chem.* **247**, 3073–81.

Warburg, O., and Christian, W. (1941). Isolation and crystallization of enolase. *Biochem. Z.* **310**, 384–412.

Watts, D. C. (1973). Creatine kinase (adenosine 5′-triphosphate-creatine phophotransferase). *The Enzymes* **8**, 383–455.

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