Properties and Reaction with Iodoacetamide of Adenosine 5'-Triphosphate-Creatine Phosphotransferase from Human Skeletal Muscle

FURTHER EVIDENCE ABOUT THE ROLE OF THE ESSENTIAL THIOL GROUP IN RELATION TO THE MECHANISM OF ACTION

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1. The purification of creatine kinase from human and monkey skeletal muscle by horizontal electrophoresis on Sephadex blocks is described. 2. The purified enzymes are shown to have similar chemical and kinetic properties to the rabbit muscle enzyme and a common mechanism is inferred. 3. Iodoacetamide has a similar apparent second-order inhibition constant with the human and rabbit enzymes, but the inhibition does not go to completion with the former. This is even more marked with the monkey enzyme, which has more reactive thiol groups, but inhibition is only about 50%. 4. Single substrates have little effect on the inhibition by iodoacetamide, but with the primate enzymes, in contrast with the rabbit enzyme, high concentrations of ADP-Mg²⁺ plus creatine convert the essential thiol group from being pH-independent into one with a normal ionization. Low concentrations of ADP-Mg²⁺ plus creatine first enhance the rate of inactivation, but cause protection as the reaction proceeds. These results are interpreted to indicate an activation of the thiol group on the subunit to which the substrates bind and a co-operatively induced decrease in the activity of the thiol group on the other subunit which lacks substrates. 5. The effects of a substrate equilibrium mixture on the rate of inhibition are essentially those of ADP-Mg²⁺ plus creatine. 6. Since no substrate combination affords significant protection to the thiol group associated with the catalytic site to which the substrates are bound, it is concluded that any mechanism involving the thiol group in a direct participation in the transition-state complex of the catalytic reaction must be abandoned unless the transition state is only a small part of the time taken for one catalytic cycle.

Most studies on mammalian creatine kinase (EC 2.7.3.2) have been on the enzyme from rabbit skeletal muscle. The enzyme from mouse muscle appears to be essentially similar in its chemical and mechanistic properties (Hooton & Watts, 1966), whereas that from ox has a similar amino acid composition with two reactive thiol groups located in similar amino acid sequences, although the overall 'fingerprint' pattern is rather different from that of the rabbit enzyme (Thomson, Eveleigh, Laws & Miles, 1968). A role for the essential thiol group per catalytic site in the mechanism of action has been suggested by Watts & Rabin (1962). The ox enzyme has not been studied, but that from chicken muscle appears to have

properties incompatible with the suggested thiol function (Hooton, 1968). From an evolutionary point of view it appears improbable that the essential thiol group has different mechanistic functions in the kinases from different vertebrates or even between vertebrates and invertebrates (Watts, 1965, 1968; Thoai, 1968).

The work now reported, which forms part of our investigation into the nature of muscular dystrophy, describes some chemical and kinetic properties of human creatine kinase that show sufficient differences from the rabbit enzyme to cast doubt on the validity of the Watts & Rabin (1962) mechanism.

Because of the difficulty of obtaining pure human creatine kinase with a high specific activity the enzyme was also purified from the monkey *Macaca mulatta*. It was found to be kinetically similar to

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the human enzyme and hence invaluable for checking some experiments. Our available information on this enzyme is also reported.

MATERIALS AND METHODS

Materials

Double-glass-distilled water was used throughout.

Substrates. Disodium ATP was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and from the Boehringer Corp. (London) Ltd., London W.5., U.K. No difference was detected between the two preparations. Other nucleotides were from Boehringer Corp. (London) Ltd. and creatine phosphate from Sigma (London) Chemical Co. Ltd. Creatine (British Drug Houses Ltd., Poole, Dorset, U.K.) was crystallized from hot 1 mM-EDTA, pH 7.5, and then from water, and finally dried at 100°C.

Buffers. For electrophoresis, tris-acetate buffer, pH 8.6, and borate buffer, pH 8.5, were prepared as described by Kumudavalli & Watts (1968). Tris-citrate buffer, pH 7.5, was prepared by dissolving 1.05 g of citric acid and 2.288 g of tris base in a final volume of 1 litre. For the determination of pH optimum tris-HCl buffers, pH 7.0, 7.5, 8.2 and 8.8 and 10.1 at 30°C, and glycine-NaOH buffers, pH 8.8, 9.15, 9.6 and 10.3 and 10.1 at 30°C, were prepared as described in Long (1961).

NN-Di-(2-hydroxyethyl)glycine (bicine) was prepared by the reaction of sodium chloroacetate with diethanolamine (Khromov & Remisov, 1953). The product was twice recrystallized from 80% (v/v) redistilled methanol, 1 mM-EDTA, pH 7.5, and water. Bicine buffers were prepared by titrating 0.5 M-NaOH with a concentrated solution of bicine to the desired pH and diluting the solution to the desired ionic strength (equal to the Na⁺ concentration) and finally rechecking the pH.

Other chemicals. These were of the best grade obtainable and, except where mentioned otherwise, were used without further treatment.

Methods

Enzyme assay. Except where mentioned otherwise the general assay procedure used was by determination of the acid-labile creatine phosphate formed in 10 min at 30°C in a total reaction mixture of 0.4 ml (Virden & Watts, 1964). If necessary, correction was made for the non-linearity of product formed as a function of enzyme concentration.

Enzyme inhibition experiments. These were carried out at 30°C in a total volume of 1 ml in bicine-NaOH buffer, pH 8.5 and 10.1, or other buffer as mentioned in the text. The reaction was started by the addition of 0.05 ml of iodoacetamide, and 0.1 ml portions were transferred immediately and after the appropriate time-intervals to 0.5 ml of ice-cold 5 mM-cysteine, pH 8.5, in the bicine buffer to stop the reaction. The enzyme activity remaining was then determined as described above. The iodoacetamide was purified as described by Watts, Rabin & Crook (1961).

RESULTS

Purification of creatine kinase. Human skeletal muscle was obtained as soon as possible, the optimum time being 1–2 h after death, and stored at

–20°C until used, usually within 1 week. Monkey skeletal muscle was dissected out immediately after the animal had been killed with anaesthetic, cooled in ice for 30 min and used at once.

Purification of the human enzyme was first attempted by the ethanol fractionation method of Kuby, Noda & Lardy (1954a) for rabbit muscle, but this method resulted in poor yields and a very low specific activity. Satisfactory purification was achieved by electrophoresis on Sephadex G-100 blocks.

Human muscle (usually less than 100 g) was extracted twice with 3 vol of cold tris-citrate buffer, pH 8.6, containing 1 mM-EDTA in an M.S.E homogenizer at 0°C. The homogenate was centrifuged at approx. 34 000 g for 30 min at 4°C, and the supernatant was decanted through EDTA-washed glass wool to remove lipid and then concentrated to approx. 1 ml by vacuum dialysis (Virden & Watts, 1964) against the same tris-citrate buffer at 3°C. The concentrated solution was centrifuged again at 34 000 g at 4°C and the supernatant used for electrophoresis.

Preparation of Sephadex block. Sephadex G-100 [Pharmacia (G.B.) Ltd., London W.13, U.K.] (6 g) was allowed to swell for 3 days in the tris-citrate buffer, pH 8.6, at 3°C and changed at intervals to remove fines. Finally the gel was allowed to settle and as much buffer as possible decanted off. The thick slurry was then poured into a Perspex frame (20 cm × 12 cm internal dimensions) resting on a clean glass plate (20 cm × 20 cm). The excess of buffer leaked out from the edge of the frame and was blotted with filter paper, leaving a firm block of Sephadex about 0.5 cm thick. A certain amount of experience is required to achieve a block of the correct consistency, as removal of too much buffer will result in the proteins migrating very slowly and the block may crack. The block was kept cold during these operations.

Application of sample and electrophoresis. The sample was applied as a band about 0.5 cm wide across the width of the block 3 cm from the cathode end, leaving 1.5–2.0 cm on either edge of the block. The Perspex frame was removed and thick filter-paper wicks soaked in borate buffer, pH 8.5, were used to connect the block to the buffer vessels, which contained the same borate buffer. The apparatus was set up in the Phaerograph (Mini 1965) electrophoresis machine with the glass plate carrying the Sephadex block resting on a thick aluminium plate, to provide optimum heat transfer to the refrigerated chamber, but electrically insulated from it by a thin sheet of polythene. Electrophoresis was carried out for 5 h at 200 V (constant voltage) and with the Phaerograph set to –3°C the temperature in the Sephadex block was maintained at 2–3°C.

After electrophoresis the block was divided into

0.5cm fractions, each of which was separately eluted with tris-citrate buffer, pH 8.6, and the Sephadex was removed by filtering through a sintered-glass funnel. The fractions of optimum enzyme activity were combined and reconcentrated by vacuum dialysis but with tris-citrate buffer, pH 7.5, and then further purified by electrophoresis again on the Sephadex block at pH 7.5 with tris-citrate buffer, pH 7.5, for the block and the borate buffer, pH 8.5, for wicks and electrode vessels. The enzyme fraction was eluted from the blocks as before, concentrated by vacuum dialysis against tris-HCl buffer, pH 8.6 and 10.1, and stored at -20°C .

Human muscle creatine kinase purified in this way gave only a single protein band when analysed by starch-gel electrophoresis, and this coincided with the position of muscle creatine kinase activity. No other creatine kinase isoenzymes could be detected. A single symmetrical protein peak was also obtained when the enzyme was passed down a Sephadex G-100 column for molecular-weight determination. The monkey enzyme was purified in the same way with similar results.

The specific activity of the human enzyme, assayed by the procedure of Kuby *et al.* (1954a), was about 0.13 unit before purification, 16.5 units after the first electrophoresis purification and 25 units after the second electrophoresis purification followed by a final dialysis against 1mm-dithiothreitol (Calbiochem, Los Angeles, Calif., U.S.A.), pH 8.5. The purified monkey enzyme had a specific activity of 89 units. These values compare with that for the rabbit enzyme of 75 units (Mahowald, Noltmann & Kuby, 1962).

Properties of human creatine kinase

Molecular weight. This was determined on a column of Sephadex G-100 by the method of Andrews (1964), with horse myoglobin, ovalbumin,

human serum albumin and rabbit creatine kinase as standards (assumed molecular weights 17800, 45000, 68000 and 83000 respectively). Four determinations gave values between 81280 and 85000 with a mean of 82530. Throughout this paper a molecular weight of 81000 is assumed for the purpose of calculation. This is because this value was first determined for the rabbit enzyme (Noda, Kuby & Lardy, 1954) and, although a higher value has recently been reliably reported (Table 1), the value of 81000 is still used as a basis for calculation so that comparisons with earlier work remain valid. In practice the difference is barely significant.

Molecular extinction coefficient. A sample of the enzyme (concentration approx. 15mg/ml) was dialysed against several changes of water made pH 8.0 with dilute ammonia. Portions of the dialysed solution were used for measurements of extinction at 280nm in a Unicam SP.800 spectrophotometer for protein determination by the biuret method (Gornall, Bardawill & David, 1949), calibrated with Armour Pharmaceutical Co. (Eastbourne, Sussex, U.K.) Standard Protein Solution, and for dry-weight determinations by freeze-drying to constant weight over phosphorus pentoxide and using an EMB1 electromicrobalance (Research and Industrial Instrument Co., London S.E.22, U.K.). The dry-weight and biuret methods gave identical results and for an assumed molecular weight of 81000 gave $\epsilon_{280}^{1\text{cm}} 7.1 \times 10^4$. This is identical with the value obtained by Noda *et al.* (1954) using glycine buffer, pH 9.8 (Table 1). The molecular extinction coefficient of the monkey enzyme was determined in a similar way (Table 1).

Nitrogen content and amino acid composition. These are compared with those of the monkey and rabbit in Tables 1 and 2.

(a) Nitrogen content (method communicated by Dr G. R. Webster, Department of Chemical Pathology, Guy's Hospital Medical School). A

Table 1. *Comparison of some properties of creatine kinases from human, monkey and rabbit skeletal muscle*

Details of methods and conditions used are given in the text. Tyrosine, tryptophan and thiol contents are calculated for an enzyme molecular weight of 81000.

	Human enzyme	Monkey enzyme	Rabbit enzyme	
Nitrogen content	16.6	—	17.0	(Noda <i>et al.</i> 1954)
$\epsilon_{280}^{1\text{cm}}$	7.1×10^4	7.95×10^4	7.1×10^4	(Noda <i>et al.</i> 1954)
Molecular weight	82500	—	83000	(Yue, Palmieri, Olson & Kuby, 1967)
Tyrosine (mol/mol of enzyme)	19.5	19.5	19.0	(Noltmann, Mahowald & Kuby, 1962)
Tryptophan (mol/mol of enzyme)	5.5	5.5	7	(Noltmann <i>et al.</i> 1962)
Thiol groups/molecule of enzyme				
Free	1.67	2.0	1.6	(Bayley & Thomson, 1967)
Total in urea	6.66	7.0	6.3	(Bayley & Thomson, 1967)
Total in guanidine hydrochloride	6.66	7.0	7.7	(Bayley & Thomson, 1967)

known amount of enzyme was digested for 1 h with 0.5 ml of a mixture containing 7 ml of concentrated sulphuric acid, 1 ml of syrupy phosphoric acid and 100 mg of potassium persulphate. The excess of acid was neutralized with 5 ml of 3.6 M-sodium hydroxide and the ammonia was determined by the phenol-hypochlorite method (Fawcett & Scott, 1960). Ammonium chloride was used to prepare a calibration curve.

(b) Tyrosine and tryptophan. These were determined by the spectrophotometric method of Beavan & Holiday (1952) with a Unicam SP.800 spectrophotometer. The absorption spectra of the native and alkali-denatured enzyme are very similar to those for other phosphagen kinases (Virden, Watts & Baldwin, 1965) with the ratio of extinction at 250 nm and 280 nm being 1:2.2.

(c) Thiol content. This was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (R. N. Emanuel Ltd., Alpertton, Middx., U.K.) as described by Ellman (1959), in sodium phosphate buffer, pH 8.0 and $I 0.003$, assuming ϵ_{420}^{cm} 13 600 for the thionitrophenate ion. Guanidine hydrochloride was prepared as described by Hooton & Watts (1966) and urea

was stored as a 10 M solution over Bio-Deminrolit (The Permutit Co. Ltd., London W.4, U.K.) mixed-bed ion-exchange resin.

(d) Amino acid composition. Samples of creatine kinase were exhaustively dialysed against water and freeze-dried before hydrolysis at 110°C with constant-boiling hydrochloric acid in vials sealed under vacuum. For the human enzyme duplicate samples were hydrolysed for 16, 46, 64 and 136 h. For the monkey enzyme triplicate samples were hydrolysed for 24 and 72 h. The human-enzyme hydrolysates were analysed on a Technicon auto-analyser and the monkey enzyme hydrolysates on a Locarte autoanalyser. Table 2 shows the best values for the nearest integral number of amino acid residues.

(e) 'Fingerprinting'. Creatine kinase (2 mg) was denatured by heating at 90°C for 2 min and then digested with 0.02 mg of trypsin ($3 \times$ cryst.; Worthington, Biochemical Corp., Freehold, N.J., U.S.A.) in 2% (w/v) ammonium hydrogen carbonate solution plus a drop of toluene (final volume 2 ml) for 24 h at 37°C. The solvent was evaporated by a stream of hot air, the residue treated with 0.25 ml of acetic acid and the mixture sublimed at 60°C. The dried peptide mixture was dissolved in 0.01 ml of

Table 2. *Amino acid compositions of human, monkey and rabbit muscle creatine kinases*

Analyses of the human enzyme were carried out by Dr A. R. Thomson, Wantage Research Laboratory, Berks., U.K.; those of the monkey enzyme were carried out with the assistance of Dr M. D. Melamed, Department of Chemical Pathology, Guy's Hospital Medical School. Data for the rabbit are from Kuby & Noltmann (1962). —, Not determined.

Amino acid	Nearest integral number of mol of amino acid residues/81 000 g of protein		
	Human enzyme	Monkey enzyme	Rabbit enzyme
Aspartic acid	73	66	83
Threonine	31	28	34
Serine	41	45	41
Glutamic acid	75	75	74
Proline	45	42	37
Glycine	66	64	62
Alanine	48	35	25
Valine	46	47	53
Methionine	19	18	19
Isoleucine	25	24	25
Leucine	64	62	70
Tyrosine	20	17	19
Phenylalanine	26	28	30
Histidine	28	—	33
Tryptophan	6	—	7
Lysine	66	55	65
Amide (NH ₃)	53	—	52
Arginine	32	32	34
Cysteine	7	—	8

Fig. 1. Comparison of the 'fingerprints' of creatine kinase from (a) human and (b) rabbit skeletal muscle. After tryptic digestion the peptides were separated on thin layers of silica gel G. Experimental details are given in the text. Peptides that are clearly different between the two species are shown in black. The peptides inside the broken lines could not be separated sufficiently accurately for precise comparisons to be made.

pyridine-acetic acid-water (10:0.4:90, by vol.) buffer, pH 6.4. Peptide separation was carried out on a thin-layer plate (20 cm × 20 cm) of silica gel G (nach. Stahl; E. Merck A.-G., Darmstadt, Germany)

Fig. 2. Optimum pH of human muscle creatine kinase in the direction of creatine phosphate synthesis at 30°C. The assay mixture (final vol. 2 ml) contained 4 mM-ATP-Mg²⁺, 32 mM-creatine, enzyme and 1 ml of tris-HCl buffer, 1.0.1 (●), or of glycine-NaOH buffer, 1.0.1 (○). The solid lines without symbols show comparable data for the rabbit enzyme (recalculated from Kuby *et al.* 1954b).

0.4 mm thick. The dried plate was lightly sprayed with the pyridine buffer and placed on the cooling plate of the Phaerograph (Mini 65) electrophoresis machine. The sample (approx. 0.5 mg) was applied, electrical connexions were made via thick pads of filter paper soaked in the same buffer and 400 V was passed for 2 h at 0–3°C. After electrophoresis the plate was dried and chromatographed at right-angles in butan-1-ol-pyridine-acetic acid-water (68:40:14:25, by vol.) as described by Wieland, Georgepoulos, Kempe & Wachsmuth (1964). The plate was dried again and the peptides were stained with ninhydrin in the usual way. Fig. 1 compares ‘fingerprints’ of human and of rabbit creatine kinase.

Kinetic properties (a). Optimum pH. This was measured only in the direction of creatine phosphate synthesis (Fig. 2). The rabbit enzyme has a similar broad pH optimum (Kuby, Noda & Lardy, 1954b) but slightly displaced towards higher pH values (Fig. 2).

(b) Michaelis Constants. These were determined in the usual way from Lineweaver & Burk (1934) plots (Table 3). In the forward reaction measured in bicine-NaOH buffer, pH 8.5 and 10.1, at 30°C the *K_m* for creatine (concentration varied between 4 mM and 16 mM) was measured in the presence of ATP-Mg²⁺, each 4 mM; that for ATP-Mg²⁺ (ratio

Table 3. Comparison of some kinetic constants for human and rabbit creatine kinase

Details of the conditions used are given in the text.

		<i>K_m</i> (mM)		References
Substrate	pH	Human enzyme	Rabbit enzyme	
ATP-Mg ²⁺ (1:1)	9.0	3.13	0.5	Kuby <i>et al.</i> (1954b)
	8.5	3.13	—	
	8.0	—	0.45	
Creatine	9.0	1.47	16.0	Kuby <i>et al.</i> (1954b)
	8.5	1.6	—	
	8.0	—	6.1	
ADP-Mg ²⁺ (1:1)	7.0	1.74	0.8	Kuby <i>et al.</i> (1954b)
	7.0	—	0.5	
Creatine phosphate	7.0	3.5	5.0	Kuby <i>et al.</i> (1954b)
	7.0	—	6.0	

		Phosphate <i>K_i</i> (mM)		Reference
Substrate varied		Human enzyme	Rabbit enzyme	
ATP-Mg ²⁺ (1:1)	8.5	62.0	—	Nihei, Noda & Morales (1961)
Creatine	8.5	40.0	—	
	9.0	—	130	
ADP-Mg ²⁺ (1:1)	7.0	35.0	—	Nihei <i>et al.</i> (1961)
Creatine phosphate	7.0	35.0	—	
	6.3	—	40	

kept 1:1 over the concentration range 0.25mM to 2.5mM) was measured in the presence of 20mM-creatine. Measurements of K_m were also made in the forward direction at pH 9.0 and 10.1, without buffer, by using the titrimetric assay of Hooton & Watts (1966) and the same concentration range of substrates.

Measurements of K_m values for the reverse reaction in tris-HCl buffer, pH 7.0 and 10.1 (Long, 1961), were determined by assaying the creatine liberated by using the method described by Rosenberg Ennor & Morrison (1956) for arginine. A creatine calibration curve was prepared and the extinction of the coloured complex measured at 535nm (Morrison, O'Sullivan & Ogston, 1961).

(c) Inhibition by inorganic phosphate. This is of interest because in the rabbit enzyme inorganic phosphate appears to compete for the site occupied by the transferred phosphoryl group in the catalytic reaction. Hence it is a competitive inhibitor for

Fig. 3. Lineweaver-Burk plots to show the inhibition of human muscle creatine kinase by orthophosphate at 30°C. When creatine phosphate was the variable substrate the ADP and $MgSO_4$ concentrations were both 4mM. When the creatine phosphate concentrations was held constant at 12mM the ADP and $MgSO_4$ concentrations were both varied so that the ADP/ Mg^{2+} ratio remained 1:1. ●, Control without phosphate; O, measured in the presence of 100mM-orthophosphate. Other experimental details are given in the text.

ATP and creatine phosphate but a non-competitive inhibitor for ADP and creatine (Nihei *et al.* 1961). A similar pattern of inhibition was found with the human enzyme. Fig. 3 illustrates data for the reverse reaction. Because inorganic phosphate interferes with the phosphate assay inhibition was determined titrimetrically at pH 9.0 for the forward reaction under conditions similar to those described above for the determination of the Michaelis constants but with 25mM-inorganic phosphate added. Inhibitor constants derived from Dixon (1953) plots are listed in Table 3.

Inhibition by iodoacetamide. The initial velocity of inhibition of human creatine kinase by iodoacetamide is proportional to inhibitor concentrations used in our experiments and followed apparent second-order kinetics with a rate constant of $4701\text{mol}^{-1}\text{min}^{-1}$ at 30°C in bicine-NaOH buffer, pH 8.5 and 10.1. This value is lower than those found for the rabbit enzyme, 600 and $8151\text{mol}^{-1}\text{min}^{-1}$ at 20°C by Hooton & Watts (1966) and $7001\text{mol}^{-1}\text{min}^{-1}$ at 20°C by O'Sullivan, Diefenback & Cohn (1966), but similar to those found for the mouse enzyme, 440 and $5201\text{mol}^{-1}\text{min}^{-1}$ (Hooton & Watts, 1966). On the other hand the purified monkey enzyme yielded an apparent second-order velocity constant of $13001\text{mol}^{-1}\text{min}^{-1}$, which indicates a much greater reactivity by the essential thiol groups of the native enzyme than for any other species so far reported.

(a) Effect of pH. Fig. 6 (control) shows that the initial velocity of enzyme inhibition is independent of pH over the range in which the enzyme shows greatest stability. Similar results were obtained in 0.05M-tris-HCl buffers adjusted to 0.05*I* and 0.1*I* with sodium chloride, showing that the phenomenon was not a feature of the buffer type or a particular ionic strength.

(b) Effects of substrates. Apart from ATP- Mg^{2+} (Fig. 4a) single substrates (substrate-magnesium sulphate complex, always in the molar ratio 1:1, is considered as a single substrate) and analogues had no effect on the rate of inhibition or the human enzyme by iodoacetamide either in bicine-NaOH buffer, pH 8.5, or in tris-HCl buffer, pH 7.0. The concentrations used were: ATP- Mg^{2+} , ADP- Mg^{2+} and AMP- Mg^{2+} , 0.25mM and 4mM; creatine, 8mM; creatine phosphate- Mg^{2+} , 1mM. Single substrates had no effect on the inhibition of the rabbit enzyme by iodoacetamide (Watts & Rabin, 1962).

With ATP- Mg^{2+} plus creatine phosphate an unusual effect was observed in that the enzyme was immediately activated by about 40% (Fig. 4b). However, the subsequent inhibition by iodoacetamide was faster, with the enzyme being 50% inhibited in 3min rather than 5min as found without substrates. This substrate combination has not been investigated with the rabbit enzyme.

Fig. 4. Effects of ATP-Mg²⁺ and of ATP-Mg²⁺ plus creatine phosphate on the rate of inhibition of human muscle creatine kinase by iodoacetamide at pH 8.5 and 30°C. For graph (a) the reaction mixture contained enzyme and iodoacetamide (0.0625 mM): ●, alone; ○, plus ATP and MgSO₄ (both 0.25 mM); Δ, plus ATP and MgSO₄ (both 4 mM). For graph (b) the reaction mixture contained enzyme and iodoacetamide (0.25 mM): ●, alone; ○, plus creatine phosphate (1 mM), ATP and MgSO₄ (both 4 mM). Experimental details are given in the Materials and Methods section.

The other dead-end complex, ADP-Mg²⁺ plus creatine, has been reported to afford strong protection to the rabbit enzyme against inhibition by iodoacetate and iodoacetamide (O'Sullivan *et al.* 1966) and against digestion by trypsin (Lui & Cunningham, 1966; Jacobs & Cunningham, 1968). The human enzyme behaves differently and the effect varies with the nucleotide-Mg²⁺ concentration. With 0.25 mM-ADP-Mg²⁺ plus 8 mM-creatine at pH 8.5 iodoacetamide enhanced the rate of inhibition during the early phase of the reaction but afforded progressively more protection as the reaction proceeded (Fig. 5a). This result was consistently obtained in six experiments with two enzyme preparations and also with a crude muscle extract (Fig. 5b). With the crude muscle extract as enzyme source the rate of inhibition was nearly

Fig. 5. Effects of ADP-Mg²⁺ plus creatine on the inhibition of human muscle creatine kinase by iodoacetamide at pH 8.5 and 30°C. For graph (a) the reaction mixture contained purified enzyme and iodoacetamide (0.0625 mM): ●, alone; ○, plus ADP and MgSO₄ (both 0.25 mM) and creatine (8 mM); Δ, plus ADP and MgSO₄ (both 4 mM) and creatine (8 mM). For graph (b) the reaction mixture contained the dialysed muscle supernatant as prepared for electrophoresis and iodoacetamide (0.0625 mM): ●, alone; ○, plus ADP and MgSO₄ (both 0.25 mM) and creatine (8 mM).

twice that of the purified enzyme and approached much more closely to zero activity. However, in the presence of the dead-end complex the inhibition curve levelled off with about 40% of the activity remaining for both enzyme preparations (Fig. 5). When the ADP-Mg²⁺ concentration was raised to 4 mM but with the same creatine concentration a large enhancement of the rate of inhibition by iodoacetamide occurred and there was now no protective phase (Fig. 5a). The effect of pH on this process was then investigated and revealed that in the presence of 4 mM-ADP-Mg²⁺ plus 8 mM-creatine the essential thiol groups had become freely ionizing with pK about 8.6 (Fig. 6).

The effect of an equilibrium mixture of substrates

Fig. 6. Plot of the apparent second-order rate constant, k_2 , for the inhibition of human muscle creatine kinase as a function of pH in the presence and absence of ADP-Mg²⁺ plus creatine at 30°C. The reaction was carried out in bicine-NaOH buffer, 10.1, at the pH values shown. ●, Enzyme plus iodoacetamide (0.0625 mM) alone; ○, enzyme plus ADP and MgSO₄ (both 4 mM) and creatine (8 mM); the iodoacetamide concentration varied from 0.0625 mM to 0.00625 mM at the higher pH values. The solid line is a calculated curve for an ionizing group pK 8.6.

was investigated by incubating either 0.25 mM- or 4.0 mM-ATP-Mg²⁺ with 8 mM-creatine and enzyme for 15 min before adding the iodoacetamide. The inhibition progress curves were similar to those obtained with ADP-Mg²⁺ plus creatine but less pronounced (Fig. 7). With the low nucleotide-Mg²⁺ concentration the initial velocity was the same as the control, but the reaction progressively deviated in the direction of protection; with the high nucleotide-Mg²⁺ concentration the rate of inhibition was slightly enhanced. These findings are again different from those obtained with the rabbit enzyme, where a substrate equilibrium mixture gave about 35% protection against inhibition (Watts & Rabin, 1962).

The effects of ADP-Mg²⁺ plus creatine and the substrate equilibrium mixture were also tested on the inhibition of the monkey enzyme by iodoacetamide. Essentially the same results were obtained as with human creatine kinase (Fig. 8), and in the presence of the dead-end complex a freely ionizing thiol group was formed (Fig. 9).

Fig. 7. Inhibition of human muscle creatine kinase by iodoacetamide in the absence and presence of an equilibrium mixture of substrates at pH 8.5 and 30°C. Each reaction mixture contained bicine buffer, pH 8.5 and 10.1, enzyme, creatine (8 mM) and either ATP plus MgSO₄ (both 0.25 mM) (○) or ATP plus MgSO₄ (both 4 mM) (Δ). The enzyme reaction was allowed to proceed to equilibrium (15 min) before the addition of iodoacetamide (0.0625 mM). ●, Control without added substrates.

DISCUSSION

The significant discovery about human creatine kinase is the markedly different way in which its substrates affect the rate of alkylation by iodoacetamide as compared with the rabbit enzyme. One would expect the enzymes from two mammals to be fairly similar in their properties, and in particular those properties concerned with the fundamental features of the mechanism of catalysis. The differences in chemical properties (Table 1) amino acid analysis (Table 2) and 'fingerprints' (Fig. 1) are no greater than would be expected to occur between two different mammalian groups. Kumudavalli & Watts (1968) have shown the human enzyme to be composed of two dissociable subunits just like that from rabbit. The 'fingerprint' evidence supports this finding.

The similarities also extend to the general kinetic behaviour (Fig. 2). Although differences exist for the Michaelis constants, that for creatine being tenfold smaller for the human enzyme and that for ATP + Mg²⁺ being sixfold larger, much less difference exists between those for the substrates for the back reaction (Table 3). This is understandable, since it is in the breakdown of creatine phosphate to replenish the ATP supply for muscle contraction that creatine kinase fulfils its main function and thus where the stabilizing influence of selection

Fig. 8. Effects of an equilibrium mixture of substrates and the dead-end complex, ADP-Mg²⁺ plus creatine, on the inhibition of monkey muscle creatine kinase at pH 8.5 and 30°C. Graph (a) shows the effect of substrate equilibrium mixtures prepared with low (O) and high (Δ) concentrations of ATP plus MgSO₄ as described in Fig. 7. Graph (b) shows the effect of the dead-end complex prepared with low (O) and high (Δ) concentrations of ADP plus MgSO₄ as described in Fig. 5. ●, Control curves obtained in the absence of substrates.

would be greatest. A lower Michaelis constant for creatine would make the enzyme more efficient in the direction of creatine phosphate synthesis when, under physiological conditions, the availability of ATP would not be a problem. Hence changes in the Michaelis constants are indicative only of evolutionary adaptation rather than any fundamental change in the catalytic mechanism. The inhibition characteristics of inorganic phosphate, which appears to act by binding to the site of phosphoryl transfer in the enzyme from both species, provides additional support for the view that the mechanism of catalysis is the same in both. How, then, are the iodoacet-

Fig. 9. Effect of pH on the apparent second-order rate constant of monkey muscle creatine kinase in the absence and presence of ADP-Mg²⁺ creatine at 30°C. ●, Control without substrates; O, plus dead-end complex as described in Fig. 6. The solid line is a theoretical ionization curve for a group with pK 8.5. Experimental details are the same as for Fig. 6.

amide inhibition differences explained, particularly when it is borne in mind that in the absence of modifiers the essential thiol groups of both enzymes show the same unusual character of having a reactivity that is independent of pH (Fig. 6).

The greatest disparity lies in the behaviour with ADP-Mg²⁺ plus creatine, which gives substantial protection to the rabbit enzyme but produces a free thiol group in the human and monkey enzymes (Figs. 6 and 9). The only reasonable inference is that the behaviour of the thiol group in the presence of this dead-end complex is not an important feature of the transphosphorylation mechanism and reflects interactions involving the species-variable parts of the enzyme. With the primate enzymes the type of interaction varies with the ADP-Mg²⁺ concentration in the reaction mixture. The low ADP-Mg²⁺ concentration provides an effective concentration of ADP-Mg²⁺ complex in the reaction mixture of 0.095 mM, as calculated from the stability constant of 4000 M⁻¹ (Morrison & Uhr, 1966), and this would on average saturate only one of the two catalytic sites on each enzyme molecule. In the reaction (Figs. 5 and 8) an initial enhancement of inactivation by iodoacetamide is followed by protection as the reaction proceeds. With the high ADP-Mg²⁺ concentration (effective concentration of ADP-Mg²⁺ complex 3.11 mM) a high proportion of both sites on each

enzyme molecule would be saturated and enhancement of inhibition occurs throughout the reaction (Figs. 5 and 8). Clearly at low ADP-Mg²⁺ concentrations the dead-end complex must activate only the essential thiol group in the subunit to which it binds and at the same time, or on alkylation, the thiol group in the substrate-free subunit becomes strongly protected, presumably as the consequence of a conformational change. A small residual activity (about 10%) after alkylation under similar conditions is found with the rabbit enzyme but was previously attributed to partial oxidation of the thiol groups (Watts *et al.* 1961). Partial oxidation could not account for the high values of residual activity observed with the very active monkey kinase (Table 1 and Fig. 8) and a conformational change seems the only reasonable inference. Further, this conclusion is compatible with the report that after maximal inhibition of the rabbit enzyme the small amount of activity left can only be destroyed by the total denaturation of the enzyme (Noda, Nihei & Moore, 1961). That such conformational changes may involve some loss of activity is suggested by Fig. 4(b) in which the other dead-end complex, ATP-Mg²⁺-creatine phosphate, facilitates activation.

An equilibrium mixture of substrates affects the rate of inhibition of the primate enzymes by iodoacetamide in a similar way to ADP-Mg²⁺ plus creatine, but to a smaller extent (Figs. 7 and 8). There is possibly a small contribution by the enzyme-ATP-Mg²⁺ complex (Fig. 4a), but the major influence is clearly the formation of the enzyme-ADP-Mg²⁺-creatine complex (Figs. 5 and 8b). With the rabbit enzyme Watts & Rabin (1962) observed protection against iodoacetamide inhibition by a substrate equilibrium mixture and used this finding as the basis for an enzyme mechanism in which the essential thiol group was protected in the transition-state complex of the reaction. They did not investigate the effect of ADP-Mg²⁺ plus creatine, and the strong protection these gave was only shown subsequently (O'Sullivan *et al.* 1966). The work reported here shows that there is now no evidence for protection of the essential thiol group in the transition-state complex of the catalytic reaction and consequently no evidence for the mechanism proposed by Watts & Rabin (1962). However, such a mechanism is not completely eliminated, because if the transition-state complex represented only a small fraction of the time taken in the total reaction pathway then the measurable protection would be very small. From this point of view the formation of dead-end complexes may be adding further confusion to an already complicated situation. It must be concluded that at the present time the nature of the catalytic mechanism is still a completely open question.

Some general evidence has already been forthcoming for conformational changes on the binding of substrates to the rabbit enzyme (Samuels, 1961; Watts, 1963; Jacobs & Cunningham, 1968). It is not surprising that such conformational changes can also be co-operative with the modification of the essential thiol group of one subunit causing the essential thiol group of the second subunit to become protected. Co-operativity appears to be much more marked in the primate kinases than in the rabbit enzyme, and this should provide a good material for further study of this phenomenon.

Although alkylation of the thiol groups was not determined quantitatively it seems inconceivable that both thiol groups per molecule could have been fully alkylated under the various conditions used for Fig. 8 and yet result in such different degrees of residual activity. However, this point has still to be checked formally.

The problem remains as to the role of the essential thiol groups. The most attractive alternative to direct participation in the catalytic mechanism is a supporting role involving the mediation of a conformational change. The studies by O'Sullivan *et al.* (1966) on the properties of the alkylated enzyme and the observation that alkylation with iodoacetamide renders the enzyme considerably less stable than when iodoacetate is used (Watts, 1966) strongly suggest that this is at least part of their function.

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