Purification and Properties of Adenosine Triphosphate–Creatine Phosphotransferase from Muscle of the Dogfish *Scylliorhinus canicula*

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1. Creatine kinase occurs in high concentration in the soluble proteins of dogfish muscle. A fourfold purification gives essentially pure enzyme but with a low specific activity. This appears to be a property of the native enzyme and not a result of the isolation procedures used. 2. The amino acid composition is similar to that of other phosphagen kinases, but the enzyme differs from mammalian creatine kinases in having four thiol groups readily reactive towards 5,5'-dithiobis-(2-nitrobenzoic acid). Titration of two thiol groups is accompanied by almost complete loss of activity. The remaining two thiol groups react at different rates, suggesting that modifying the third thiol group affects the reactivity of the fourth thiol group. 3. The enzyme is markedly protected against inactivation by iodoacetamide by MgATP or MgADP. Addition of creatine to MgADP decreases protection, but the further addition of Cl^- restores protection to the original value. The quaternary MgADP–creatine–enzyme–nitrate complex protects very strongly as is found for the rabbit enzyme. The involvement of the conformational state of the enzyme in such effects is discussed. 4. Creatine kinase from both dogfish and rabbit is equally sensitive to urea denaturation. Urea protects the dogfish enzyme by about 9% against inhibition by iodoacetamide. 5. The formation of a hybrid between the dogfish and rabbit enzymes *in vitro* has been demonstrated. 6. At high substrate concentrations the dogfish enzyme shows apparent ordered kinetics. The effect of temperature on $V_{\max}$ and the Michaelis constants for MgATP and creatine were determined. These and changes in the apparent activation energy suggest that limited adaptation has occurred commensurate with physiological need.

Although the creatine kinase (ATP–creatine phosphotransferase, EC 2.7.3.2) from rabbit muscle has been intensively studied and a number of investigations have been made on the enzyme from various tissues of other warm-blooded vertebrates, those of cold-blooded animals have been largely neglected. Brief investigations have been made of the frog and turtle muscle creatine kinases, and among the fishes some molecular weights (Moreland *et al.*, 1967) and electrophoretic patterns have been determined (Scopes & Gosselin-Rey, 1968; Gosselin-Rey *et al.*, 1968), including those of the dogfish enzyme.

It was considered that the dogfish enzyme would repay further study, since this animal is able to tolerate a fairly wide range of temperatures (5–17°C, and an even wider range of temperatures for short periods of time). Further, its tissues are unusual in containing about 2% (w/v) urea (0.33M), thereby offering further possibilities for adaptation at the molecular level.

While this work was in progress, the isolation and some properties of creatine kinase from muscle of the carp, *Cyprinus carpio*, were described by Gosselin-Rey

& Gerday (1970). Their investigation of a freshwater fish has proved a useful complement to our own studies on a marine elasmobranch.

Materials and Methods

Materials

ATP (disodium salt) and ADP (trisodium salt) were obtained from Boehringer Corp. (London) Ltd. London W.5, U.K. Creatine and buffer salts were obtained and purified as described by Kumudavalli *et al.* (1970) and other compounds as described by Milner-White & Watts (1971). Sephadex G-100 was obtained from Pharmacia (G.B.) Ltd., London, W.13, U.K., and Whatman DE 52 pre-swollen cellulose ion-exchanger from H. Reeve Angel and Co. Ltd., London E.C.4, U.K.

Enzyme assay

Creatine kinase activity was measured by determining the acid-labile creatine phosphate formed in 10 min at 30°C in a total reaction volume of 0.4 ml, as described by Virden & Watts (1966). For most

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measurements product formation was first-order with respect to enzyme concentration. In a few experiments with high enzyme concentration it was necessary to correct for the non-linearity of product formation as a function of enzyme concentration. This was done by means of a calibration curve in which the amount of product formed as a function of enzyme concentration had been determined.

Iodoacetamide-inhibition experiments

These were carried out at 30°C in a total volume of 0.5 ml in the presence of glycylglycine buffer, pH 8.0, 1 0.02, dithiothreitol, 0.1 mM, and sodium acetate to make the total ionic strength 0.1. When nucleotides were added these were dissolved in the above buffer before being adjusted to pH 8.0. The reaction was started by adding iodoacetamide in 0.02 ml. At time-intervals ranging from 0.5 to 20 min, portions (0.05 ml) were transferred to 0.25 ml of an ice-cold solution containing cysteine, 8 mM, and creatine, 80 mM, pH 8.5. After standing for 5 min the sample was then equilibrated at 30°C for 5 min and assayed for creatine kinase activity by adding 0.1 ml of ATP-MgSO₄ mixture (Virden & Watts, 1966).

Other methods are described in the text.

Purification of dogfish creatine kinase

Live dogfish (*Scylliorhinus canicula* L.) were obtained from the Laboratory of the Marine Biological Association of the United Kingdom,

Plymouth, Devon, U.K. On arrival at the laboratory the animals were killed, and the back muscles were freed from gut and skin and either used at once or stored at -20°C until required. No appreciable loss of creatine kinase activity was found in a sample that had been stored for 9 months before extraction, compared with a freshly extracted sample. Fresh or deep-frozen white muscle (200–500 g) was cut into small pieces and homogenized in a Waring Blendor with 2 ml of ice-cold 1 mM-EDTA solution (pH 8.0)/g of muscle. A microspatula tip of phenylmethane-sulphonyl fluoride dissolved in 0.2 ml of methanol was added to guard against proteolytic inactivation. The homogenate was centrifuged at 15000g for 30 min at 4°C and the residue discarded. The supernatant was made approx. 1 mM with dithiothreitol and the protein fraction precipitated within the range 35–55% (v/v) with acetone precooled to -20°C was collected by centrifugation as above. The precipitate was dissolved in 1 mM-EDTA, pH 8.0, in one-tenth of the initial extracting volume and then concentrated two- to seven-fold by vacuum dialysis (Watts & Moreland, 1970) by using the same EDTA buffer containing 0.1 mM-dithiothreitol. The enzyme was then further purified by electrophoresis on a block of Sephadex G-100 for 3½ h at 30 mA with a tris-citrate, pH 8.6, buffer system as described by Kumudavalli *et al.* (1970) or by chromatography at 4°C on DE 52 pre-swollen cellulose ion-exchanger that had been washed from fines and equilibrated with 1.38 mM-tris-1 mM-EDTA buffer, pH 7.8, and packed to give a column (2.5 cm × 85 cm). The extract was

Fig. 1. Purification of dogfish creatine kinase by chromatography on DE 52 DEAE-cellulose ion-exchanger

The 55% (v/v) acetone precipitate, treated as described in the text, was applied to the column in 2 ml volume and eluted with a NaCl gradient after any protein that did not bind to the column had first been washed through. Fractions (5 ml) were collected. —, Protein (E_{280}); ●, enzyme activity (μmol of phosphocreatine synthesized/min) per fraction; ○, specific activity; ----, concn. of NaCl. Other details are given in the text.

Table 1. *Summary and comparison of the procedures used for the purification of dogfish creatine kinase*

Full experimental details are given in the text.

Stage	Chromatography				Electrophoresis			
	Protein (mg)	Total activity ($\mu\text{mol/min}$)	Specific activity ($\mu\text{mol/min per mg}$)	Recovery (%)	Protein (mg)	Total activity ($\mu\text{mol/min}$)	Specific activity ($\mu\text{mol/min per mg}$)	Recovery (%)
Muscle	500×10^3				200×10^3			
Crude supernatant extract	8016	25090	3.13	100	2256	9024	4.00	100
35% Acetone supernatant	—	—	—	—	1776	8792	4.95	98
55% Acetone precipitate	3140	16170	5.15	64	794	5876	7.4	65
Chromatography								
Part of the concentrated extract applied to column	233	962	4.13					
Combined concentrated fractions (166–192)	40	524	13.1	54				
Electrophoresis								
Part of the concentrated extract used for electrophoresis					476	3520	7.4	
Combined concentrated fractions					82	1279	15.6	36

Table 2. *Amino acid composition of dogfish creatine kinase*

The methods used are described in the text and the values found are compared with those from carp (Gosselin-Rey & Gerday, 1970) and rabbit (Noltmann *et al.*, 1962).

Amino acid	Nearest integral number of residues/mol		
	Dogfish	Carp	Rabbit
Aspartic acid	83	91	83
Threonine	33	38	34
Serine	32	41	41
Glutamic acid	73	72	74
Proline	36	31	37
Glycine	63	63	62
Alanine	30	27	25
Valine	48	50	53
Methionine	20	21	19
Isoleucine	32	33	25
Leucine	70	64	70
Tyrosine	19	15	19
Phenylalanine	26	31	30
Lysine	61	61	65
Histidine	36	26	33
Arginine	38	32	34

elution was carried out by running on to the column 500ml of the equilibrating buffer from a magnetically stirred aspirator to which 250ml of 50mM-NaCl dissolved in the equilibrating buffer and contained in a 250ml conical flask was connected by a siphon. Fractions (5ml) were collected and those with the highest enzyme activity were pooled and reconcentrated by vacuum dialysis. An elution profile showing the constant specific activity of the enzyme peak is presented in Fig. 1. Table 1 summarizes and compares the two methods of purification.

Both preparations gave single bands of protein corresponding to the position of enzyme activity when analysed by starch-gel electrophoresis as described by Watts & Moreland (1970). Additional bands with a greater electrophoretic mobility appeared after storage for several weeks at -18°C . These were greatly decreased by the presence of dithiothreitol. The specific activity was determined by the method of Kuby *et al.* (1954a) and yielded 6.0 and 6.5 Kuby units for the chromatographic and electrophoretic preparations respectively. Moreland *et al.* (1967) report a molecular weight for the dogfish enzyme of 83000. For the purpose of comparison the value of 81000, widely used for the rabbit enzyme, has been assumed since this falls within the experimental error of the method of determination.

Results

Chemical properties

These were studied on a creatine kinase preparation purified by the electrophoretic method.

applied to the column in 2ml and then washed with 400ml of the equilibrating buffer to remove proteins that were not adsorbed on to the column. Gradient

Amino acid composition. This is compared with the amino acid composition of carp creatine kinase in Table 2 and was determined by analysis on a Locarte

Table 3. *Determination of the number of thiol groups in dogfish creatine kinase by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) in the absence and presence of guanidine hydrochloride*

The reaction was carried out at room temperature in 4mM-sodium phosphate buffer, pH8.0.

Concn. of enzyme (μM)	No. of reactive thiol groups/81 000 g of enzyme	Total no. of thiol groups/81 000 g of enzyme (measured in 6M-guanidine hydrochloride)
0.99	3.85	—
1.73	4.23	—
3.66	4.02	6.03

Fig. 2. *Titration of the number of catalytic sites in dogfish creatine kinase with 5,5'-dithiobis-(2-nitrobenzoic acid)*

The initial enzyme concentration used for reaction with the reagent was $140 \mu\text{g/ml}$ and in the subsequent assay was $8.7 \mu\text{g/ml}$. Additions of the reagent were arranged so that the final concentrations in the reaction mixture were 0.4, 0.8, 1.4 and $2.0 \mu\text{M}$. Other details are described in the text. Each point is the mean of two to five determinations. Activity is expressed as a percentage of that obtained in the absence of reagent.

autoanalyser of samples hydrolysed for 24 and 72h by the method of Moore *et al.* (1958). The column system and elution conditions were as described by Leslie & Cohen (1970). The values for valine, leucine and isoleucine were taken from the 72h analysis only and those for threonine and serine by extrapolation to 0h hydrolysis time.

The amino acid composition of dogfish creatine kinase is generally similar to those from warm-blooded vertebrates and yields a partial specific volume of 0.733 ml/g as compared with 0.735 ml/g obtained for the rabbit enzyme (Noltmann *et al.*, 1962).

Tyrosine and tryptophan content. These were determined by the method of Beaven & Holiday (1952), in which the protein is dissolved in 0.1M-NaOH and extinctions are measured at 280 and 294nm, and by the method of Edelhoch (1967) in which the sample is dissolved in 6M-guanidine hydrochloride and extinctions are measured at 280

Fig. 3. *Semi-log plots showing the effect of the dead-end complex (creatine plus MgADP) with and without added anions on the inhibition of creatine kinase by iodoacetamide at pH8.0 and 10.1 at 30°C*

The dead-end complex consisted of 1mM-ADP, 10mM-magnesium acetate and 40mM-creatine. The initial enzyme concentration reacting with iodoacetamide was $140 \mu\text{g/ml}$ and in the subsequent enzyme assay, $17.5 \mu\text{g/ml}$. o, No substrate added; ●, plus dead-end complex; △, plus dead-end complex and 10mM-KCl; ▲, plus dead-end complex and 10mM-KNO₃. Other details are given in the text and in Table 4.

Table 4. *Effects of substrates and other modifiers on the inhibition of dogfish creatine kinase by iodoacetamide at 30°C*

Where more than one experiment was carried out under a particular set of conditions, the mean value for the apparent second-order rate constant (K) and the percentage protection is given. The percentage protection is the protection obtained as compared with a control experiment carried out at the same time and under the same conditions but with modifiers omitted. Other experimental details are given in the text.

No. of expts.	Concn. of iodoacetamide (mM)	Additions	K ($l \cdot mol^{-1} \cdot min^{-1}$)	Protection (%)
2	0.125	Nil	662	
1	0.25	Nil	645	
1	0.5	Nil	625	
1	0.125	ADP (1 mM), Mg acetate (10mM)	330	49
1	0.125	ATP (4mM), Mg acetate (10mM)	295	55
2	0.125	Substrate equilibrium mixture*	300	54
3	0.125	ADP (1 mM), Mg acetate (10mM), creatine (40mM)	433	32
1	0.125	ADP (1 mM), Mg acetate (10mM), creatine (40mM), NaCl (10mM)	330	49
1	0.125	ADP (1 mM), Mg acetate (10mM), creatine (40mM), NaNO ₃ (10mM)	95	86
2	0.125	Urea (340mM)	590	9
1	0.125	ADP (1 mM), Mg acetate (10mM), creatine (40mM), urea (340mM)	295	55
2	0.125	Substrate equilibrium mixture*, urea (340mM)	245	62

* The substrate equilibrium mixture was prepared by allowing ATP (4mM), magnesium acetate (10mM) and creatine (40mM) to react with the enzyme for 30min before addition of the iodoacetamide.

Fig. 4. *Comparison of the effect on the activity of dogfish and rabbit muscle creatine kinase of assaying for enzyme activity in the presence of various concentrations of urea*

○, Dogfish creatine kinase; ●, rabbit creatine kinase. The enzyme, in 0.4ml, was exposed to the urea concentrations shown for 10min at 30°C before adding 0.1ml of MgATP to start the assay at the same temperature.

and 288nm. Measurements were made with a Unicam SP.800 spectrophotometer and, per 81000g of protein, gave 17.4 tyrosine and 6.3 tryptophan residues by the method of Beaven & Holiday (1952) and 18.5 tyrosine and 7.6 tryptophan residues by the method of Edelhoch (1967).

A further estimation of tyrosine is obtained (Edelhoch, 1967) by making the guanidine hydrochloride solution alkaline to ionize the phenol group and then comparing extinctions at 295 and 300nm. This gave an average value for tyrosine of 18.2 residues/molecule. In general, the method of Edelhoch (1967) gave very close agreement with the value for tyrosine determined by amino acid analysis (Table 2) and for this reason the higher value for tryptophan was also preferred.

Reactive and total thiol groups. These were determined by using 5,5'-dithiobis-(2-nitrobenzoic acid) (approx. 1mM) as described by Ellman (1959), assuming $E_{412}^{1\%1\text{cm}} = 13600 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the 2-nitro-5-thiobenzoate ion in 4mM-sodium phosphate buffer, pH8.0. Total thiol groups were determined by measurements of E_{412} in 6M-guanidine hydrochloride.

With the native enzyme in aqueous solution at room temperature, three thiol groups reacted very quickly and a fourth had reacted in 5–10min. The results are summarized in Table 3.

Fig. 5. Hybridization of dogfish and rabbit muscle creatine kinase

The hybridization procedure was carried out as described in the text. Samples were analysed by horizontal starch-gel electrophoresis in a tris-citrate discontinuous buffer system, pH 8.6 (Watts & Moreland, 1970), in the cold-room at 1–4°C for 5 h at 2.6 mA/cm width of gel (constant current). (a) Unhybridized mixture of dogfish and rabbit enzymes; (b) hybridized mixture; (c) dogfish; (d) rabbit muscle creatine kinase.

General enzymic properties

All measurements were made on an enzyme sample purified by the electrophoretic technique.

Number of thiol groups titrated by 5,5'-dithiobis-(2-nitrobenzoic acid) related to activity loss. The enzyme was titrated with dilute 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of sodium glycylglycinate buffer, pH 8.0, I 0.02, containing 0.08 M sodium acetate to give a total ionic strength of 0.1. When the reaction had gone to completion, as judged by following E_{412} , a sample was assayed for residual enzyme activity. The results are shown in Fig. 2, where each point is the mean of two to five determinations. Extrapolation to zero activity gives a value of 2.14 thiol groups reacted/81000 g of enzyme.

Inhibition by iodoacetamide. The inhibition of dogfish creatine kinase by iodoacetamide was found

to be proportional to the inhibitor concentration (Table 4) and over the early part of the reaction followed pseudo-first-order kinetics, as indicated by linear semi-log plots. However, as shown by the plot in Fig. 3 without added substrates, progressive deviation from linearity occurred as the enzyme became more than 50% inhibited, and complete inhibition could not be achieved. The average value for the apparent second-order rate constant for the linear phase of the reaction from a number of determinations at 30°C in sodium glycylglycinate buffer, pH 8.0, I 0.1, was $650 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{min}^{-1}$. Dithiothreitol (0.1 mM) was also present in all reaction mixtures to stabilize the enzyme.

(a) Effects of substrates in the presence of acetate ions. When acetate was the only small anion present in the reaction mixture the addition of either MgATP or MgADP gave significant protection against iodoacetamide inhibition (Table 4). The further addition of creatine to give either the ternary dead-end complex (enzyme-creatine-MgADP) or an equilibrium mixture of substrates did not increase the protection over that obtained with the magnesium-nucleotide alone and with the ternary dead-end complex the protection was substantially decreased (Table 4).

(b) Effects of chloride and nitrate ions. NaCl or NaNO₃ alone added to the enzyme did not affect the rate of inhibition by iodoacetamide, but when added to the ternary dead-end complex (enzyme-creatine-MgADP) a marked increase in the degree of protection was obtained (Fig. 3). The effect was greatest with NO₃⁻, which resulted in almost complete protection, but with Cl⁻ the degree of protection was only restored to that found with MgADP alone. The rate constants and percentage protection obtained in these experiments are listed in Table 4.

(c) Effect of urea. The addition of urea, stored as a stock 10 M solution over Bio-Deminrolit mixed-bed resin, at concentrations similar to those found in elasmobranch tissues, resulted in slight but experimentally significant protection against iodoacetamide inhibition (Table 4). Urea also enhanced the protection by the ternary dead-end complex and the substrate equilibrium mixture (Table 4).

Effect of urea on enzyme activity. Rabbit and dogfish creatine kinase were exposed to various concentrations of urea for 10 min at 30°C in 0.4 ml of a mixture containing creatine, *NN'*-di-(2-hydroxyethyl)glycine-NaOH buffer, pH 8.5, and dithiothreitol. The enzyme activity remaining was determined by adding 0.1 ml of MgATP in glycylglycine buffer, pH 8.5, I 0.1, to start the reaction. The final concentrations in the assay mixture were: creatine, 37.5 mM; *NN'*-di-(2-hydroxyethyl)glycine, 12.5 mM; ATP, 4 mM; MgSO₄, 4 mM; dithiothreitol, 1 mM; glycylglycine, I 0.02. In Fig. 4 the results for the two enzymes have been normalized, and there is no

Fig. 6. Lineweaver-Burk plots showing the effect of varying one substrate concentration at several fixed concentrations of the second substrate in glycylglycine buffer, pH 8.5 and 1.02 at 30°C

(a) The MgATP concentration, maintained at a constant ratio of 1:1, was varied with the creatine concentrations fixed at: ○, 10 mM; ●, 12.5 mM; ▲, 16.7 mM; △, 25 mM; ■, 50 mM. (b) The creatine concentration was varied with the MgATP concentrations fixed at: ○, 0.89 mM (1.0 mM-ATP and MgSO₄); ●, 1.20 mM (1.33 mM-ATP and MgSO₄); △, 1.84 mM (2.0 mM-ATP and MgSO₄); ▲, 3.77 mM (4.0 mM-ATP and MgSO₄). In both (a) and (b) the enzyme concentration was 2.4 μg/ml.

difference in their stability towards increasing concentrations of urea.

Hybridization of dogfish and rabbit creatine kinase. The dogfish and rabbit creatine kinases have sufficiently different electrophoretic mobilities on a starch gel to allow the detection of a hybrid if this could form the enzymes of the two species. Dissociation of a mixture of the two enzymes in 8M-urea and the recombination of their subunits by dialysing away the urea under vacuum dialysis was carried out as described by Kumudavalli & Watts (1968). Starch-gel electrophoresis of the dialysed concentrated mixture revealed the presence of a new band of creatine kinase activity with the intermediate mobility expected of a hybrid between the two species (Fig. 5). Staining for protein with Amido Black revealed that a new protein band corresponded with the new band of enzyme activity.

Kinetic properties

These were investigated in the direction of phosphocreatine synthesis with an enzyme preparation purified by the chromatographic method, and all velocities are expressed as μmol of phosphocreatine synthesized/min per mg of protein. The MgATP complex was assumed to be the true substrate for the

enzyme (Noda *et al.*, 1960) and the concentration of the complex in each assay mixture was computed by using the stability constant for ATP of 77000 (O'Sullivan & Perrin, 1961). Lines were fitted to the data by using an Olivetti P203 desk computer programmed for linear regression analysis with equal statistical weighting on all points.

Effect of substrate concentration. Fig. 6 shows as Lineweaver-Burk plots the effect on the initial velocity of the enzyme reaction at 24°C of varying in turn the concentration of each substrate in the presence of several fixed concentrations of the second substrate. In these experiments the total ATP and Mg²⁺ concentrations were kept equal. The results show that for both substrates a series of essentially parallel lines are obtained, suggesting that for the conditions used the reaction could follow an ordered mechanism. The secondary plots derived from Fig. 6 of 1/(intercept) against 1/[substrate] are shown in Fig. 7. These yield identical limiting maximum velocities of 27.1 μmol/min per mg and limiting Michaelis constants for creatine and MgATP of 41.5 and 1.75 mM respectively.

Effect of temperature on the kinetic parameters. The effect of temperature from 0 to 40°C was investigated on the apparent *K_m* values for creatine and MgATP. For the determinations of the *K_m* values for creatine

the ATP and MgSO_4 concentrations were both kept constant at 12.5 mM while the creatine concentration was varied between 8.3 and 40 mM (Fig. 8). For the determination of the K_m values for MgATP the creatine concentration was kept constant at 50 mM while the ATP and MgSO_4 concentrations were kept equal and varied between 0.89 and 4.0 mM (Fig. 9). The K_m values for creatine and MgATP as a function of temperature are compared with those of other

Fig. 7. Effect of substrate concentration on the observed maximum enzyme velocity when the second substrate is saturating

(a) The concentration of creatine is saturating and that of MgATP is varied. (b) The concentration of MgATP is saturating and that of creatine is varied. Results are derived from the plots in Fig. 6.

workers in Table 5 and the effect on $V_{\max}$ is shown in Fig. 10.

Arrhenius plots derived from these data are shown in Fig. 11. In both cases, when either creatine or MgATP is the variable substrate, there is a discontinuity in the slopes at about 7°C. Calculated activation energies above 7°C were 6800 cal/mol (28400 J/mol) and 5900 cal/mol (24700 J/mol) with creatine and MgATP as the variable substrates respectively. Below 7°C the activation energy increases sharply. A precise value is not possible from the few points obtained but the line drawn in for a computed activation energy of 20000 cal/mol (83600 J/mol) is probably not an unreasonable estimate.

Discussion

The concentration of creatine kinase in dogfish muscle is about twice that of the rabbit muscle but the specific activity is very much less. The assay devised by Kuby *et al.* (1954a) for measuring specific activity, which has proved of great value for comparative purposes, is less satisfactory for the fish enzyme because, although the K_m for ATP is not very different, that for creatine (approx. 50 mM) is about three times that for the rabbit enzyme under the conditions used (24 mM-creatine, 1 mM-ATP). However, even allowing for this difference, the specific activities obtained (13–15.5) are only about one-fifth of that for the rabbit enzyme. Gosselin-Rey & Gerday (1970), using a spectrophotometric assay, found that carp creatine kinase had a specific activity about one-third that of a rabbit enzyme preparation, but unfortunately the specific activity of the latter in Kuby units is not given.

The low specific activity of the dogfish enzyme appears to be a genuine property, since when isolated by two different methods similar specific activities were obtained (Table 1) and there was no obvious heterogeneity on starch-gel electrophoresis. This is further supported by the determination of two thiol groups/molecule essential for activity (a long-recognized creatine kinase character, and which also excludes the possibility of activity loss by oxidation). Inactivation by proteolysis has not been entirely excluded, although this possibility was guarded against by the inclusion of phenylmethanesulphonyl fluoride in the homogenizing medium. Also, crude dogfish muscle extracts are not particularly unstable in the absence of an inhibitor for proteolytic enzymes.

Reactive thiol groups

The rate of inhibition of dogfish creatine kinase by iodoacetamide, yielding an apparent second-order rate constant of 650 litre·mol⁻¹·min⁻¹, is about the same as that found with the rabbit enzyme (Milner-

Fig. 8. *Lineweaver-Burk plots showing the effect of temperature on dogfish creatine kinase at pH 8.5 when the creatine concentration is varied and the ATP/MgSO₄ concentration is held at 12.5 mM*
The enzyme concentration in the assay was 6.2 μg/ml.

Fig. 9. *Lineweaver-Burk plots showing the effect of temperature on dogfish creatine kinase at pH 8.5 when the ATP/MgSO₄ concentration (maintained at 1:1) is varied and the creatine concentration is held at 50 mM*
The enzyme concentration in the assay was 6.2 μg/ml.

Fig. 10. Effect of temperature on the maximum velocity of dogfish creatine kinase at pH 8.5

○, Concentration of creatine saturating; ●, concentration of MgATP saturating. Results are derived from Figs. 8 and 9.

White & Watts, 1971). The presence of four reactive thiol groups/molecule (Table 3) is quite different, as the rabbit enzyme has only two. However, titration with 5,5'-dithiobis-(2-nitrobenzoic acid) reveals that two of these are essential for activity (Fig. 2) and react much faster than the remaining two, whose significance for catalysis must therefore remain unknown. The slowly reacting thiol groups, presumably one on each subunit, also react at markedly different rates from each other, suggesting that there is a co-operatively induced conformational change upon alkylation of the third thiol group/molecule that renders the fourth thiol group on the other subunit less reactive. This would explain the observation of three thiol groups/molecule found with the carp enzyme (Gosselin-Rey & Gerday, 1970) if in that species the fourth thiol group were rendered totally inactive by modification of the first three. The inhibition curve of dogfish creatine kinase in the absence of modifiers (Fig. 3) also suggests that modification of a thiol group on one subunit can affect the reactivity of the essential thiol group on the second subunit. Similar observations have been made for the two essential thiol groups of creatine kinases of chicken heart (Hooton, 1968), human muscle (Kumudavalli *et al.*, 1970), dystrophic, but not normal, chicken muscle (Roy *et al.*, 1970) and ox brain (Atherton *et al.*, 1970). The last group of workers found that the extent of the alkylation depended on the enzyme concentration in the reaction mixture and suggested that the degree of interaction of the subunits was a concentration-dependent phenomenon. Such

Fig. 11. Arrhenius plots of dogfish creatine kinase activity (a) with MgATP as the variable substrate and (b) with creatine as the variable substrate

The velocities are obtained from the intercepts on the ordinates in Figs. 8 and 9. The line drawn in for temperatures below 8°C is that calculated for an activation energy of 20000 cal (83 600 J). The vertical lines delimited by horizontal bars indicate ± 1 S.D.

changes in protein conformation and subunit interaction offer a rational explanation of why complete loss of activity by chemically modifying the essential thiol groups of any creatine kinase has not proved possible. The observed residual activity more probably results from a failure of complete reaction of all the thiol groups rather than from the completely modified enzyme retaining some activity. Thus the fish enzymes appear to be unusual only in that they possess more than two easily reacting thiol groups. In this they resemble the arginine kinase of lobster (Virden & Watts, 1966) and the glycocyamine kinase of *Nephtys coeca* (Kassab *et al.*, 1967), a feature that may reflect the fact that the investigations were carried out at temperatures above the normal physiological range.

A particular feature of rabbit creatine kinase is that it is strongly protected against inhibition by iodoacetamide by the presence of the substrates that form a dead-end complex, creatine and MgADP (O'Sullivan *et al.*, 1966). Milner-White & Watts (1971) showed that this protective ternary complex also depended on the presence of a planar anion or a halide ion smaller than I^- . Hence the complex was

Table 5. Comparison of the effect of temperature on the Michaelis constants for creatine, ATP and MgATP with the creatine kinase from dogfish and rabbit

Temperature (°C)	K_m (mM)				
	Dogfish		Rabbit		
	Creatine	MgATP	Creatine	ATP	MgATP
0	—	1.2	—	0.1*	0.3*
1	33	—	—	—	—
3	—	—	10–100†	0.3*	0.1†
4	—	—	—	0.5‡	0.3‡
7	52	—	—	—	—
8	—	0.8	—	—	—
16	42	1.5	—	—	—
24	53	1.5	—	—	—
30	—	—	16§	0.5§	—
	—	—	10.7	—	0.4¶
	—	—	19	—	0.45¶
32	55	2.3	—	—	—
38	—	—	19†	0.6†	—
40	167	2.3	—	—	—

* Kuby *et al.* (1962) by inactivation kinetics.

† Kuby *et al.* (1962) by equilibrium dialysis.

‡ Kuby *et al.* (1962) by gradient sedimentation.

§ Kuby *et al.* (1954b) from Lineweaver–Burk plots.

|| Milner-White & Watts (1971) from Lineweaver–Burk plots.

¶ Nihei *et al.* (1961) from Lineweaver–Burk plots.

really a quaternary one; NO_3^- caused almost complete protection and Cl^- about 80%. With NO_3^- , similar results were obtained with the dogfish enzyme (Table 4). However, Cl^- behaved differently, and this is associated with a behaviour not found with the rabbit enzyme, that MgATP or MgADP alone affords considerable protection (about 50%) against inhibition. Addition of MgADP plus creatine decreases protection whereas the addition of MgADP, creatine and Cl^- to form the quaternary dead-end complex with the enzyme only restores protection to 50% (Table 4). Hence, although the picture with NO_3^- conforms to the pattern found with the rabbit enzyme, that with Cl^- does not. Milner-White & Watts (1971) concluded that protection against alkylation results from a conformational change in the enzyme induced by the MgADP–creatine–enzyme–anion complex. Thus the probable explanation is that NO_3^- is a good mimic of the planar phosphoryl group in an intermediate stage of the transphosphorylation reaction and consequently give almost complete protection (Milner-White & Watts, 1971). On the other hand, Cl^- is only a poor mimic so that the associated conformational changes in the enzyme induced by this quaternary dead-end complex to form the precise geometry of the catalytic

site ready for transphosphorylation are no greater than can be induced by MgADP alone.

Comparison of the iodoacetamide-inhibition patterns of creatine kinase from rabbit (Milner-White & Watts, 1971) and human (Kumudavalli *et al.*, 1970) with that from dogfish indicates that, although the essential properties of the reactive thiol group are a constant feature of these enzymes, the type of protection observed with various substrate combinations can be very variable.

Evidence is accumulating that substrate protection is always associated with a conformational change in the enzyme. The nature of this conformational change is not known, but the accompanying change in the apparent reactivity of the essential thiol group might be regarded as a sensitive index of this process reflecting the slightly different ways in which the enzymes from different species achieve the same catalytic end.

The relationship between the distribution and reactivity of the enzyme thiol groups and catalytic function poses interesting structural problems, for the amino acid composition of carp and dogfish creatine kinases (Table 2) are not significantly different from those of other creatine kinases (Watts, 1971) or, indeed, from arginine kinase. Further, dogfish and rabbit creatine kinases readily form an

active hybrid (Fig. 5). Clearly the overall shape of the subunits and disposition of their combining sites may be strongly conserved in evolution, even though the total number of thiol groups and their reactivity towards alkylating agents may vary.

It has been suggested (Koffler, 1957) that the thermostability of proteins in organisms living at high temperatures (above 60°C) could be due to increased hydrophobic bonding in the protein. Bigelow (1967) has attempted to quantify this idea by defining a 'hydrophobicity index' for each protein under comparison. This is calculated from the percentage amino acid residue composition of the protein and a weighting factor having the dimensions of free energy for each residue. Bigelow (1967) found that the hydrophobicity index was higher by 10% in thermophilic proteins. From the present results (Table 2) and those summarized by Watts (1971) it is possible to calculate the hydrophobicity indices for phosphagen kinases from a variety of species living in different environments. These are (in cal/mol with the equivalent in J/mol in parentheses): for the creatine kinases of dogfish, 1084 (4530), carp, 1061 (4440), rabbit, 1074 (4490), and human, 1074 (4490); for the arginine kinases of two species of lobster, *Homarus vulgaris*, 1108 (4630) and *Homarus americanus*, 1084 (4530). By this criterion it is clear that adaptation of these species to a narrow or broad temperature band over the range 5–37°C is not accompanied by any obvious structural adaptation of the phosphagen kinase. Similarly, the dogfish enzyme shows no obvious adaptation for working in an environment containing urea (Fig. 4), although a physiological concentration of urea had a slight but experimentally significant general protecting effect against iodoacetamide inhibition (Table 4).

Enzyme kinetics

The kinetic pattern of a series of parallel lines obtained in Lineweaver–Burk plots when one substrate is varied at several fixed concentrations of the second substrate suggests that dogfish creatine kinase could proceed by an ordered mechanism (Cleland, 1967). Rabbit creatine kinase, on the other hand, is concluded to function via a rapid equilibrium-random mechanism (Morrison & James, 1965). However, Smith & Morrison (1969), in a discussion of the kinetic mechanism of arginine kinase from the sea crayfish *Jasus verreauxi*, inferred from the data of Uhr *et al.* (1966), point out that parallel initial velocity patterns may result when the substrates are varied over concentration ranges that are high relative to the values of their Michaelis constants. With the arginine kinase from another species of crayfish it was found that kinetic patterns compatible with a rapid equilibrium-random mechanism were obtained when lower substrate concentrations were used. Neverthe-

less, both arginine kinases gave exchange reactions suggesting that at least part of the reaction flux occurred via a Ping Pong sequence. The present work was also carried out with substrate concentrations that are high relative to the Michaelis constants because these conditions give good values for initial-velocity measurements by using the phosphate assay at timed intervals in the reaction. In the light of the above discussion it would clearly be premature to draw any conclusion as to the nature of the kinetic mechanism of dogfish creatine kinase and a more detailed investigation is indicated.

In this work it has been assumed that, as with the rabbit enzyme, ATP^{4-} does not cause measurable inhibitions at the concentrations present in the assay mixture (Noda *et al.*, 1960). However, correction should be made for inhibition caused by SO_4^{2-} that is competitive with ATP and added as the counterion to Mg^{2+} . Assuming that the K_i for SO_4^{2-} is 6 mM (Noda *et al.*, 1960) and the K_m for MgATP^{2-} is 1.75 mM (from Fig. 7a), then an approximate correction can be calculated from the relationship:

$$V_{\text{true}} = V_{\text{obs.}} \cdot \frac{1 + \frac{s}{K_m} + \frac{i}{K_i}}{1 + \frac{s}{K_m}}$$

where s and i are the concentrations of substrate and inhibitor respectively for each initial-velocity measurement. For the Lineweaver–Burk plots with variable MgATP^{2-} concentrations this correction gave lines essentially parallel to those shown in Fig. 6(a) but with the $V_{\text{max.}}$ and apparent K_m values increased by about 25% and 30% respectively. The secondary plot (Fig. 7b) had a slightly decreased slope and yielded a limiting $V_{\text{max.}}$ of 35.7 $\mu\text{mol/min}$ per mg and a limiting K_m for creatine of 50 mM. Lineweaver–Burk plots with creatine as the variable substrate (Fig. 6b) had a slightly decreased slope after correction, with $V_{\text{max.}}$ being progressively increased from about 10% at the lowest MgATP^{2-} concentrations to 22% at the highest used. The apparent K_m values for creatine were not affected. The slope of the corresponding secondary plot (Fig. 7a) was not altered, but the limiting $V_{\text{max.}}$ was again increased to 35.7 $\mu\text{mol/min}$ per mg and the limiting K_m for ATP to 2.3 mM. Thus, under the conditions used, when ATP and MgSO_4 were added to the assay mixture in a 1:1 ratio the apparent effect of SO_4^{2-} is to decrease the K_m for ATP by about 30%, decrease $V_{\text{max.}}$ by 10–25% and leave the K_m for creatine not significantly affected. These estimates are probably maximal, since anions appear to have less effect on dogfish creatine kinase than on the rabbit enzyme (Table 4), and although significant for the individual experiments do not affect the overall kinetic picture. Recently a higher value of 30 mM has been reported for the K_i for SO_4^{2-} (Milner-White & Watts, 1971).

The correction obtained with this value falls within the experimental error of the method. Consequently no corrections were applied to the data for Figs. 8 and 9, particularly in the absence of knowledge about the effect of temperature on the inhibition by SO_4^{2-} .

The effect of temperature on K_m and $V_{\max}$ was studied by using the range of substrate concentrations that yielded the apparent ordered kinetics. Surprisingly, these concentrations lie within the normal physiological range since, assuming a 70% tissue-water space, the creatine concentration in dogfish muscle could rise to 55 mM (Vyncke, 1970). The ATP concentration in dogfish muscle has not been reliably determined, but in cod muscle, for example, it is approx. 8.5 mM (Dyer *et al.*, 1966). Because of the high K_m value for creatine even these conditions do not completely saturate the enzyme with substrates and this is revealed in Fig. 10, where the $V_{\max}$ values are generally lower when determined in the presence of a fixed creatine concentration. The low point at 40°C clearly results from the sudden twofold increase in the K_m value for creatine that occurs near this temperature (Table 5). Up to 25°C, $V_{\max}$ changes with the usual Q_{10} of 2. Above this the pattern is abnormal, perhaps reflecting conformational changes in the enzyme. However, the Arrhenius plot is linear over this range (Fig. 11) and only shows a sharp discontinuity somewhere below 8°C, when the apparent activation energy shows an approximately threefold increase. Thus it would seem that the physiological range of temperature tolerance is reflected by changes in the properties of creatine kinase when the range is exceeded. Only limited comparison with rabbit creatine kinase can be made, since a comparable study on that enzyme has yet to be carried out. Kuby *et al.* (1954b) reported the much higher values of 11100 cal/mol (46400 J/mol) and 13600 cal/mol (54700 J/mol) for the activation energy of the forward and reverse reactions respectively, as compared with 5900 cal/mol (24600 J/mol) and 6800 cal/mol (28400 J/mol) for the dogfish enzyme in the forward direction only, but neither the creatine nor the phosphocreatine concentrations used by Kuby *et al.* (1954b) were near saturating. Further, for the rabbit enzyme there is no sharp increase in the K_m value for creatine above 30°C (Table 5), so that some physiological adaptation to temperature in the two species does appear to be indicated.

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