Biochimica et Biophysica Acta, 410 (1975) 99—114

BBA 67639

KINETIC STUDIES AND EFFECTS OF ANIONS ON CREATINE PHOSPHOKINASE FROM SKELETAL MUSCLE OF RHESUS MONKEY (*MACACA MULATTA*)

W. RICHARD CHEGWIDDEN and D.C. WATTS

Department of Biochemistry and Chemistry, Guy's Hospital Medical School, London SE1 9RT, (U.K.)

(Received June 2nd, 1975)

Summary

A purification procedure for creatine kinase (EC 2.7.3.2) from muscle of the monkey *Macaca mulatta* is described. The final preparation has a specific activity of 135—170 μequiv H^+/mg protein per min at 30°C and a yield of approx. 0.5 g/kg muscle.

Assuming equilibrium kinetics, synergistic binding of substrates at one catalytic site is found for both the forward and back reactions. Kinetic constants for the binding of each substrate to the free enzyme and the enzyme-second substrate complex are determined and are compared with those for the enzyme from other species.

Inhibition by small anions is determined in the presence of different combinations of substrates and products. SO_4^{2-} inhibits by simple competitive inhibition and probably binds at the site of the transferrable phosphoryl group. Inhibition by NO_3^-, NO_2^-, SCN^- and Cl^- is more complex and these ions are suggested to mimic the transferrable phosphoryl group in a planar transition-state complex. These anions stabilize the dead-end complex, enzyme-creatine-MgADP, which lacks the transferable phosphoryl group. The effects of these anions on the dissociation constants of the enzyme-substrate complexes is reported and is in accord with the above hypothesis.

The dead-end complex in the absence of anion does not protect the essential thiol group against inhibition by iodoacetamide. Addition of NO_3^- or Cl^- to the dead-end complex or a substrate equilibrium mixture without anion confers protection. The essential thiol group is inhibited by iodoacetamide at a rate which is essentially independent of pH over the normal stability range of the enzyme. Contrary to our previous report this pH independence is not altered by the presence of dead-end complex, creatine plus MgADP, in the presence or absence of anion or in the presence of a substrate equilibrium mixture. It is inferred that the 'essential' thiol group of the monkey enzyme has essentially the same properties as that of the rabbit enzyme. In consequence, the infer-

ences made about the role of this group based on our previous work on the moneky enzyme are no longer valid. The present findings are compatible with the essential thiol group playing a conformational role in the catalytic process.

Introduction

The first complete kinetic analysis of creatine kinase (adenosine-5′-triphosphate : creatine phosphotransferase, EC 2.7.3.2) from rabbit muscle was carried out by Morrison and James [1] and Morrison and Cleland [2], who concluded that the reaction mechanism was of the random order, rapid equilibrium type. Since the discovery that Cl^- used in their buffer system, may affect the values of kinetic constants obtained [3], only the enzymes from ox, measured in the presence of acetate ions [4] have received similar attention. The present work, also using acetate, extends the survey to include creatine kinase from muscle of the rhesus monkey, *Macaca mulatta.*

Milner-White and Watts [5], using rabbit muscle creatine kinase, divided anions into three classes on the basis of the type of effect that they had on the enzyme. Alone in class I was acetate which reversibly activated the forward reaction. Class II comprised small anions, such as NO_3^- or Cl^-, which, they suggested, inhibited by forming a stable enzyme-creatine-anion-MgADP complex, which mimicked a transition-state complex. Class III comprised larger anions such as SO_4^{2-}, which could not form the quaternary complex, but inhibited by competing with the phosphorylated substrate.

The class II anion-stabilised enzyme-creatine-MgADP complex was also found to protect the enzyme from inhibition by iodoacetamide [5]. The reactive group on the enzyme was considered to be a cysteine side chain with the property that the rate of alkylation by iodoacetamide was essentially independent of pH [6—8].

Whether the pH-independent nature of the thiol changed in the presence of substrate was not investigated at the time, but in 1970, Kumudavalli et al. [9] suggested that, in the presence of the dead-end complex, the human and rhesus monkey muscle enzymes showed a pH-dependent profile for thiol reactivity. It may be inferred from this observation that the cysteine group is not a contact amino acid in the catalytic process. For this reason the phenomenon has been reinvestigated. No evidence was found to support the idea that the thiol could become pH-dependent.

Materials and Methods

Water distilled twice from all-glass apparatus was used throughout. CO_2-free water was prepared by boiling twice-distilled water for 10 min and cooling it in a stoppered vessel fitted with a CO_2-trap.

ATP (disodium salt) was obtained from the Boehringer Corp. (London) Ltd, London W5, U.K. and ADP from Miles-Serevac (PTY) Ltd, Maidenhead, Berks., U.K.

Creatine was obtained from B.D.H. Chemicals Ltd, Poole, Dorset, U.K.,

and was recrystallised from hot 1 mM EDTA, pH 7.5, and from water and dried in the oven.

Phosphocreatine and cysteine were obtained from the Sigma (London) Chemical Co., Ltd, London SW6, U.K.

EDTA, magnesium acetate and sodium acetate and all other sodium salts were AnalaR grade and thioglycollic acid was laboratory grade, all obtained from B.D.H.

Iodoacetamide (B.D.H.) was recrystallised five times from 50% aqueous ethanol.

Ethanol (B.D.H. AnalaR grade) was redistilled after adding 2 g/l solid KOH. Acetone was refluxed with successive quantities of $KMnO_4$ until the violet colour persisted, dried with anhydrous $CaSO_4$, filtered and redistilled.

N,N-di(hydroxyethyl)glycine (bicine) was prepared by the method of Khromov and Remisov [10] by the reaction of sodium chloroacetate with diethanolamine. After recrystallising several times from 80% redistilled methanol it was recrystallised from 1 mM EDTA and finally from water.

Tris and $(NH_4)_2SO_4$ were B.D.H. AristaR grade.

Standard acid and alkali. All water used in preparation and dilution of standard acid and alkali was CO_2-free.

Constant boiling HCl was prepared by the method of Vogel [11] and accurately diluted to about 12 mM for use in assaying the enzyme back reaction (synthesis of ATP from phosphocreatine).

Saturated NaOH solution was prepared and stored in a polythene bottle fitted with a guard tube containing CO_2-absorbing soda asbestos. The saturated solution was accurately diluted to about 12 mM for use in assaying the forward reaction and stored similarly. It was standardised by titration with 12 mM HCl using the pH-stat assay assembly.

Enzyme purification. Skeletal muscle was taken from a freshly killed rhesus monkey and immediately placed in an ice/water mixture. It was then cut into small pieces, minced and homogenized twice (2 × 10 s with an interval of >1 min for cooling) in a Waring blendor with ice-cold 1 mM EDTA/0.1 M KCl/5 mM thioglycollic acid adjusted to pH 9.0 with NaOH, using 2 ml solution for every 1 g muscle. The extract was centrifuged at 5400 × *g* for 30 min at 4°C and the pellet was discarded.

Powdered anhydrous $(NH_4)_2SO_4$ was slowly added to the supernatant with constant stirring at <4°C to give a concentration of 250 g/l of original supernatant. The stirring was continued for 15 min after the final addition, after which the mixture was centrifuged at 15 000 × *g* for 20 min at 4°C. The precipitate was discarded and powdered $(NH_4)_2SO_4$ was added to the new supernatant, this time adding 130 g/l of supernatant.

After centrifuging the preparation at 15 000 × *g* for 20 min, the precipitate was extracted twice with 0.07 M magnesium acetate/5 mM thioglycollic acid adjusted to pH 9.0 with NaOH in volumes equal to firstly 6% and secondly 4% of the volume of the initial extract supernatant. The preparation was centrifuged at 33 000 × *g* for 15 min at 4°C after each extraction and the supernatants from each centrifugation were combined.

Ethanol, chilled to −20°C, was added slowly to the extract with constant stirring at <4°C to give a final ethanol concentration of 50% (v/v). Again the

stirring was continued for 15 min after the final addition.

After centrifugation at 33 000 × *g* for 15 min at 4°C, ethanol at −20°C was added to the supernatant by the same technique as used above to give a final ethanol concentration of 57% (v/v). The preparation was centrifuged at 33 000 × *g* for 15 min and the precipitate was redissolved in 5 mM bicine/10 mM thioglycollic acid adjusted to pH 8.7 with NaOH and vacuum dialysed against two changes of the same buffer to a concentration of 10 mg/ml.

The enzyme preparation was then dialysed against two changes of 5 mM bicine/5 mM thioglycollic acid, pH 8.7, followed by five changes of 5 mM bicine/NaOH plus 10^{-3} to 10^{-4} M dithiothreitol (concentration not critical).

The purified enzyme solution was stored in a deep freeze at −20°C, in aliquots of 0.5 ml. The yield of purified enzyme was approx. 0.5 g/kg fresh muscle.

The enzyme assay system. The enzyme activity was measured using a semi-automatic pH-stat assay system comprising a combined electrode type GK 2321C, a pH meter type 26, a titrator TTT11 and a 0.25-ml volume autoburette type ABU 1c, all from Radiometer, Copenhagen and a Smith's Desk Type Servoscribe Potentiometric Recorder. The autoburette, designed to give a mechanical signal, was modified by addition of a potentiometric device, to give an electrical signal which could be fed into the recorder.

The assays were performed in a total volume of 2.0 ml, thermostated at 30°C and magnetically stirred.

The forward reaction (phosphocreatine synthesis) was measured at pH 8.6 using 12 mM CO_2-free NaOH as titrant. The back reaction was measured at pH 8.0 using 12 mM HCl as titrant.

The reaction mixture routinely used for the forward reaction consisted of 40 mM creatine, 4 mM ATP, 5 mM magnesium acetate, 1 mM mercaptoacetic acid and 100 mM sodium acetate. The sodium acetate increased the specific activity by up to 25% and it was omitted from the reaction mixture when effects of different anions were being examined. The concentration of free Mg^{2+} was held at 1.0 mM as recommended by Cleland [12]. The assays were started by adding 0.1 ml of an enzyme solution suitably diluted in 5 mM mercaptoacetate/5 mM bicine/NaOH buffer, pH 8.70.

The reaction mixture used routinely for the back reaction consisted of 10 mM phosphocreatine, 1.25 mM ADP, 2.0 mM magnesium acetate and 1.0 mM mercaptoacetic acid. These conditions gave a final MgADP concentration of 1.0 mM and a free Mg^{2+} concentration of 1.0 mM (calculated using the stability constant for MgADP of 4000 M^{-1} determined by Morrison et al. [13]). For the back reaction the enzyme was diluted in 5 mM cysteine/NaOH, pH 7.90. These substrate concentrations gave almost linear progress curves, except when there was strong product inhibition. The initial slope of the progress curves was taken as a measure of the initial velocity of the reaction.

In experiments where the nucleotide concentration was varied, the magnesium acetate concentration was also varied in order to keep the free Mg^{2+} concentration at 1.0 mM.

For the forward reaction a low blank value of never more than about 2% of the maximum velocities measured was obtained in the absence of enzyme and this was attributed to CO_2 absorption. For the back reaction there was no

measurable blank. Care was taken at all times to ensure that the titrant delivery tube was very close to the electrode and that stirring of the reaction mixture was very efficient.

Starch gel electrophoresis. Horizontal starch gel electrophoresis was carried out in the cold room at 4°C using the method and staining techniques described by Watts and Moreland [14].

Iodoacetamide inhibition experiments. Most of these experiments were carried out at 30°C in a total volume of 1.0 ml in 25 mM bicine/NaOH buffer, pH 8.60, and I = 0.172. The enzyme was incubated with all the experimental components, with the exception of iodoacetamide, for at least 15 min after which a 0.1-ml sample, representing time 0, was transferred to 0.9 ml of 5 mM cysteine/NaOH, pH 8.7. The reaction was started by adding 0.1 ml iodoacetamide solution in water, thus restoring the total reaction volume to 1.0 ml and reducing the enzyme concentration by 10%. Aliquots of 0.1 ml were rapidly transferred at timed intervals into 0.9-ml samples of cysteine/NaOH as above and instantly mixed well. The samples were assayed using the pH-stat assay system.

Experiments using a very low enzyme concentration (0.29 mg/ml) were performed in 12.5 mM bicine/NaOH buffer, pH 8.60 (I = 0.00858) and samples of 0.2 ml were transferred from the iodoacetamide reaction mixture into 0.8 ml of 6.25 mM cysteine/NaOH solution, pH 8.7.

Results

Purity of the enzyme preparation. Starch gel electrophoresis of the purified monkey muscle creatine kinase revealed a single discrete band of creatine kinase activity and a single corresponding protein band.

The specific activity of different purified enzyme samples varied within the range 135—170 μequiv H^+ released/mg protein per min. Most of the enzyme used in the experiments reported here possessed activity towards the higher end of this range. This range of values compares quite well with creatine kinase isolated from other sources [15].

Protein measurement. Routinely, the absorption of the enzyme at 280 nm was measured using a Unicam SP 800 spectrophotometer. An extinction coefficient of $8.85 \cdot 10^4$ M was used. This was obtained by correlating Lowry estimations at several enzyme concentrations with a calibration curve obtained using bovine serum albumin as a standard protein (Underhill, J.A., personal communication).

Kinetic studies. Figs 1A, 1B and 2A, 2B show the effects on the initial velocity of the enzyme reaction of varying in turn the concentration of each substrate in the presence of several fixed concentrations of the other substrate for the forward and back reactions, respectively. Secondary plots of the vertical intercepts and the slopes of these curves plotted against each fixed concentration of the second substrate are shown in Figs 1C, 1D and 2C, 2D. Assuming equilibrium kinetics, dissociation of the enzyme with the second substrate were calculated by the method of Florini and Vestling [17]. These constants are defined and their values compared with those obtained for creatine kinase from other sources in Tables I and II.

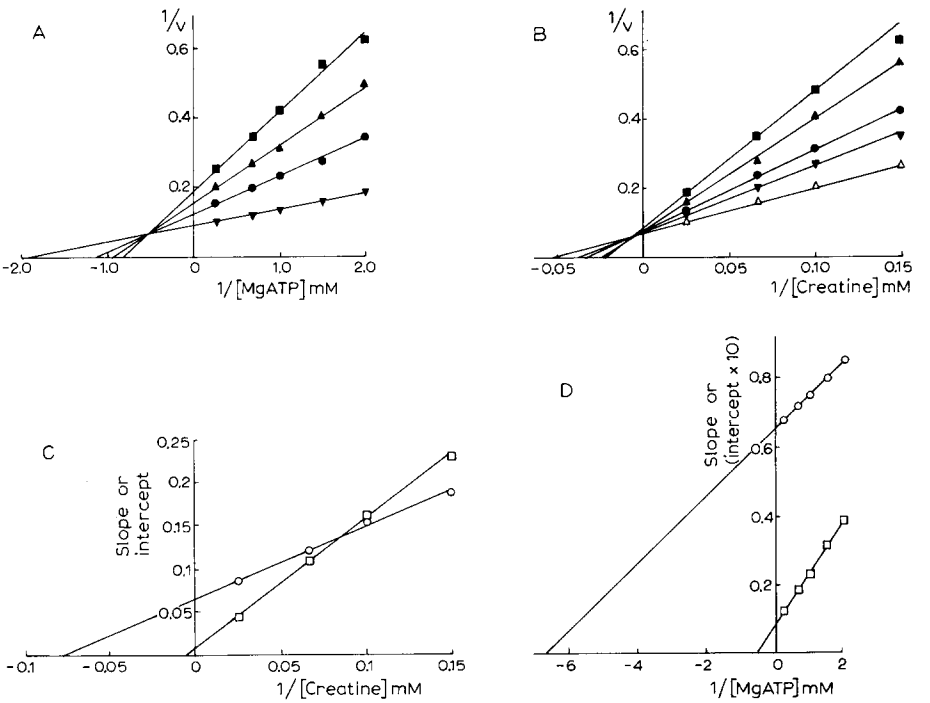

Fig. 1. Kinetics of the forward reaction. V is the relative initial velocity of the enzyme-catalysed reaction. (A) Effect of varying the MgATP concentration in the presence of several fixed concentrations of creatine. The fixed concentrations of creatine were: ■, 6.67 mM; ▲, 10 mM; ●, 15 mM; ▼, 40 mM. (B) Effect of varying the creatine concentration in the presence of several fixed concentrations of MgATP. The MgATP concentrations were: ■, 0.5 mM; ▲, 0.67 mM; ●, 1.0 mM; ▼, 1.5 mM; △, 4.0 mM. (C) Secondary plots of the data in (A), showing the variation of maximum initial velocity (○) and gradient of the primary plots (□) as a function of creatine concentration. (D) Secondary plots of the data in (B), showing the variation of maximum initial velocity (○) and gradient of the primary plots (□) as a function of MgATP concentration. Other conditions were as described in Materials and Methods.

According to this kinetic treatment, the constants thus obtained are related by the equations:

$$K_a \cdot K_{b'} = K_{a'} \cdot K_b \text{ and } K_p \cdot K_{q'} = K_{p'} \cdot K_q$$

In the present study, the calculations of $K_a \cdot K_{b'}$ and $K_a \cdot K_{a'}$ give 22.6 and 22.8, respectively, and those of $K_p \cdot K_{q'}$ and $K_{p'} \cdot K_q$ give 6.5 and 6.2, respectively.

Effects of inorganic anions on the forward reaction. Table III shows the degree of inhibition produced by several different inorganic anions at 100 mM concentration. This inhibition was found to be reversible by dilution.

Normal progress curves were generally found to be linear over the first 2—3 min of the enzyme-catalysed reaction in the presence of 0.1 M sodium acetate or in the absence of added anions. In the presence of NO_2^-, NO_3^- or Cl^-, progress curves were noticeably curved during the first few minutes of the reaction. However, the larger, non-planar anions, SO_4^{2-} and SCN^- inhibited without noticeably affecting the linearity of the progress curves.

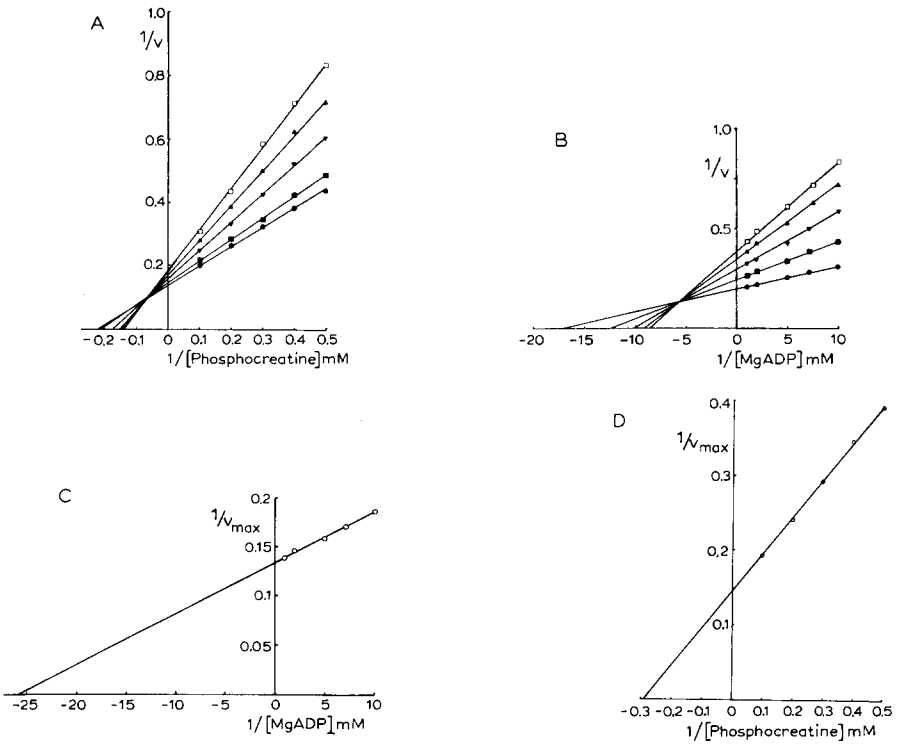

Fig. 2. Kinetics of the back reaction. V is the relative initial velocity of the enzyme-catalysed reaction. (A) Effect of varying the phosphocreatine concentration in the presence of several fixed concentrations of MgADP. The fixed concentrations of MgADP were: □, 0.1 mM; ▲, 0.14 mM; ▲, 0.2 mM; ■, 0.5 mM; ●, 1.0 mM. (B) Effect of varying the MgADP concentration in the presence of several fixed concentrations of phosphocreatine. The fixed concentrations of phosphocreatine were: □, 2.0 mM; ▲, 2.5 mM; ▼, 3.3 mM; ■, 5.0 mM; ●, 10.0 mM. (C) Secondary plots of the data in (A), showing the variation of maximum initial velocity as a function of MgADP concentration. (D) Secondary plots of the data in (B), showing the variation of maximum initial velocity as a function of phosphocreatine concentration. Other conditions were as described in Materials and Methods.

Fig. 3D shows, as Lineweaver-Burk plots, that SO_4^{2-} is simply competitive with MgATP, while Cl^- and NO_3^- are not. The apparent K_i values for these three anions are listed in Table IV.

The product MgADP acts as an inhibitor competitive with the substrate MgATP and this inhibition is enhanced in the presence of Cl^- (Fig. 3B) and even more so in the presence of NO_2^- (Fig. 3C). In the presence of SO_4^{2-} no such enhancement occurs (Fig. 3A).

Inhibition by Cl^-, NO_2^- or SO_4^{2-} is not competitive as compared with acetate when MgATP is the variable substrate.

In these experiments the reaction progress curves became increasingly more curved as the initial MgATP concentration was lowered. Traces were more curved in the presence of NO_2^-.

The other product, phosphocreatine, inhibited much less than MgADP and appeared not to be simply competitive with creatine. Furthermore, the addition of Cl^- caused no greater enhancement of phosphocreatine inhibition than was found on addition of acetate ions.

TABLE I

COMPARISON OF KINETIC CONSTANTS FOR THE FORWARD REACTION FOR THE MONKEY SKELETAL MUSCLE ENZYME WITH THOSE FROM OTHER SOURCES

The constants for the monkey skeletal muscle enzyme are derived from the data presented in Fig. 1. The constants for the calf smooth muscle enzyme were determined by Focant and Watts [16], for the calf skeletal muscle and brain enzymes by Jacobs and Kuby [4] and for the rabbit enzyme (in the presence of Cl^-) by Morrison and James [1]. E, enzyme; Cr, creatine.

Kinetic constant	Reaction	Value of constant (mM)				
		Monkey skeletal muscle	Calf skeletal muscle	Calf smooth muscle	Calf brain	Rabbit skeletal muscle
K_a	E-Cr ⇌ E+Cr	154	53	2.20	29	15.6
K_b	E-MgATP ⇌ E+MgATP	1.79	0.97	0.75	0.93	1.2
K'_a	Cr-E-MgATP ⇌ E-MgATP+Cr	12.75	21	0.58	3.7	6.1
K'_b	Cr-E-MgATP ⇌ E-Cr+MgATP	0.147	0.78	0.20	0.13	0.48
K_a/K'_a		12.1	2.52	3.8	7.85	2.56
K_b/K'_b		12.2	1.24	3.75	7.15	2.5

TABLE II

COMPARISON OF KINETIC CONSTANTS FOR THE BACK REACTION FOR THE MONKEY SKELETAL MUSCLE ENZYME WITH THOSE FROM OTHER SOURCES

The constants for the monkey skeletal muscle enzymes are derived from the data presented in Fig. 2. The constants for the calf enzymes were determined by Jacobs and Kuby [4] and for the rabbit enzyme (in the presence of Cl^-) by Morrison and James [1]. E, enzyme; PCr, phosphocreatine.

Kinetic constant	Reaction	Value of constants (mM)			
		Monkey skeletal muscle	Calf skeletal muscle	Calf brain	Rabbit skeletal muscle
K_p	E-PCr ⇌ E+PCr	16.7	45	20	8.6
K_q	E-MgADP ⇌ E+MgADP	0.177	0.17	0.12	0.17
K'_p	PCr-E-MgADP ⇌ E-MgADP+PCr	3.5	23	2.0	2.9
K'_q	PCr-E-MgADP ⇌ E-PCr+MgADP	0.039	0.094	0.01	0.05
K_p/K'_p		4.76	1.96	10	3.0
K_q/K_q		4.5	1.81	12	3.4

TABLE III

INHIBITION OF CREATINE KINASE ACTIVITY IN THE FORWARD DIRECTION BY VARIOUS ANIONS

Assay conditions were as described in Materials and Methods. Data for the rabbit muscle enzyme are taken from ref. 5.

Anion (100 mM)	Inhibition (%)	
	Monkey muscle	Rabbit muscle
Nil	0	0
$NaNO_2$	72	81
$NaNO_3$	63	67
NaSCN	36	—
Na_2SO_4	33	12
NaCl	24	27

TABLE IV

INHIBITOR CONSTANTS FOR CLASS II ANIONS

These constants were derived from Fig. 3D. Conditions were as described in Materials and Methods.

Variable substrate	Inhibitor	Apparent K_i (mM)
MgATP	$NaNO_2$	11
MgATP	Na_2SO_4	23
MgATP	NaCl	64

TABLE V

EFFECTS OF ANIONS ON MICHAELIS CONSTANTS AND INHIBITOR CONSTANTS FOR SUBSTRATES AND PRODUCTS OF THE CREATINE KINASE REACTION IN THE FORWARD REACTION

The concentration of MgADP added as an inhibitor was 0.161 mM (calculated from the stability constant found by Morrison and Uhr [18]). The concentration of phosphocreatine added as an inhibitor was 6 mM. In experiments where the ATP concentration was varied, the magnesium acetate concentration was also varied such that it was always present in a 1 mM excess of the ATP concentration.

Variable substrate	Product inhibitor	Anion (0.1 M)	Apparent K_m (mM)	Apparent K_i (mM)
MgATP	—	acetate	0.35	—
MgATP	—	NO_2^-	1.73	—
MgATP	—	Cl^-	1.3	—
MgATP	—	SO_4^{2-}	2.0	—
MgATP	MgADP	Acetate	—	0.27
MgATP	MgADP	Cl^-	—	0.16
MgATP	MgADP	NO_2^-	—	0.13
MgATP	MgADP	SO_4^{2-}	—	0.51
Creatine	—	acetate	13.5	—
Creatine	—	Cl^-	9.4	—
Creatine	phosphocreatine	acetate	—	45
Creatine	phosphocreatine	Cl^-	—	44

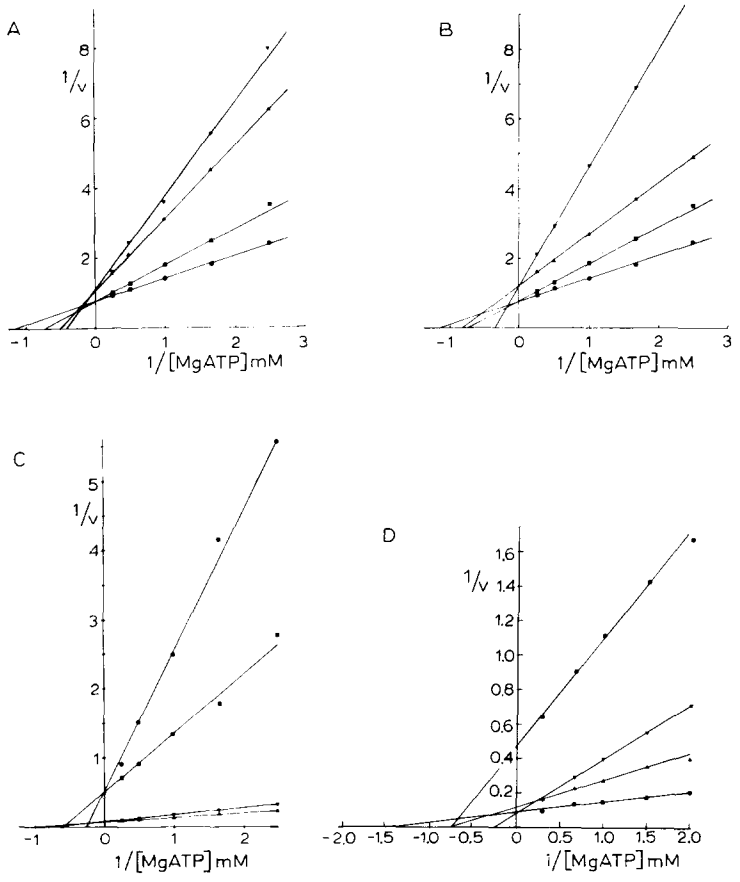

Fig. 3. Effects of anions on substrate and product-inhibitor binding in the forward reaction. V is the relative initial velocity of the enzyme-catalysed reaction. (A) Comparison of the inhibition of MgADP plus either acetate or sulphate: ●, plus 0.1 M sodium acetate; ■, plus 0.1 M sodium acetate and 0.161 mM MgADP; ▲, plus 0.1 M Na_2SO_4; ▼, plus 0.1 M Na_2SO_4 and 0.161 mM MgADP. The enzyme concentration was 1.1 μg/ml. Other conditions were as described in Materials and Methods section. (B) Comparison of the inhibition by MgADP plus either acetate or chloride: ●, plus 0.1 M sodium acetate; ■, plus 0.1 M sodium acetate and 0.161 mM MgADP; ▲, plus 0.1 M NaCl; ▼, plus 0.1 M NaCl and 0.161 mM MgADP. Other conditions were as described in (A). (C) Comparison of the inhibition by MgADP plus either acetate or nitrite: ▲, plus 0.1 M sodium acetate; ▼, plus 0.1 M sodium acetate and 0.161 mM MgADP; ■, plus 0.1 M $NaNO_2$; ●, plus 0.1 M $NaNO_2$ and 0.161 mM MgADP. Other conditions were as described in (A), except that the enzyme concentration was 5.4 μg/ml in the assays performed in the presence of NO_2^-. (D) Comparison of the effects of different anions on varying the MgATP concentration: ●, no additions; ▲, plus 0.1 M NaCl. ▼, plus 0.1 M Na_2SO_4; ■, plus 0.1 M $NaNO_2$. Other conditions were as described in (A), except that a different enzyme preparation was used.

The kinetic constants derived from these data are listed in Table V.

It may be noted that Cl^- and SO_4^{2-} all increased the apparent K_m for the substrate MgATP and NO_2^- and Cl^- decrease the apparent K_i for the product inhibitor MgADP, whilst SO_4^{2-} increases it by comparison with the constants obtained in the presence of acetate above. A similar comparison shows that Cl^- decreases the apparent K_m for creatine, but has no effect on the apparent K_i for phosphocreatine.

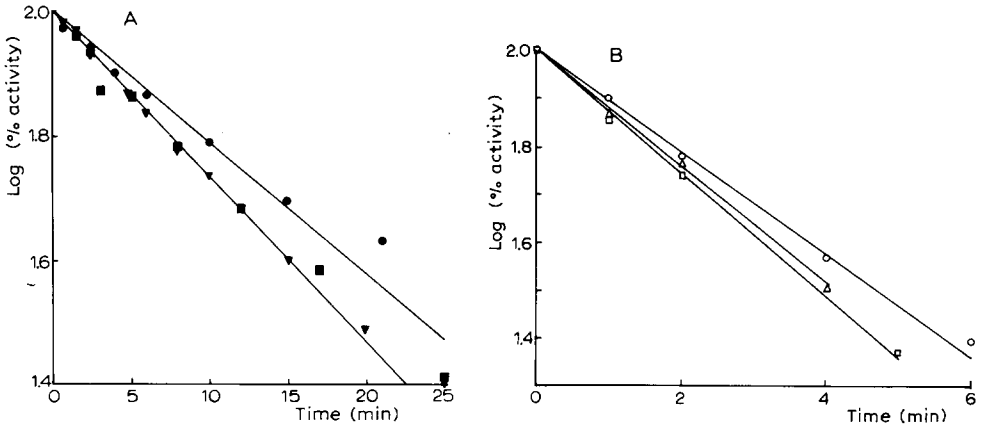

Fig. 4. Inhibition of creatine kinase by iodoacetamide. (A) Effect of the dead-end complex and the substrate equilibrium mixture on the rate of inhibition at 30°C: ▼, no added substrates; ■, plus dead-end complex (40 mM creatine, 1 mM ADP and 10 mM magnesium acetate); ●, plus substrate equilibrium mixture (40 mM creatine, 4 mM ATP and 10 mM magnesium acetate). The iodoacetamide concentration was 0.0625 mM and the enzyme concentration was 0.47 mg/ml. (B) Effect of pH on the rate of inhibition in the presence of dead-end complex at 30°C: ○, pH 7.6; △, pH 8.6; □, pH 9.4. The iodoacetamide concentration was 0.36 mM and the enzyme concentration was 0.47 mg/ml.

Inhibition by iodoacetamide. The inhibition of creatine kinase by iodoacetamide was found to be first order with respect to both the enzyme and the inhibitor. However, since under the conditions employed the iodoacetamide was greatly in excess and its concentration changed negligibly over the course of an experiment, the inhibition appeared to follow first-order kinetics.

In the absence of modifiers, a second-order rate constant for the inhibition reaction of 960 $M^{-1} \cdot min^{-1}$ at pH 8.6 and 30°C was obtained (Fig. 4A).

Fig. 4A shows that while the presence of an equilibrium mixture of substrates protected by about 19% against iodoacetamide inhibition (calculated from the second-order rate constants), the presence of creatine and MgADP in the absence of a small anion afforded no protection.

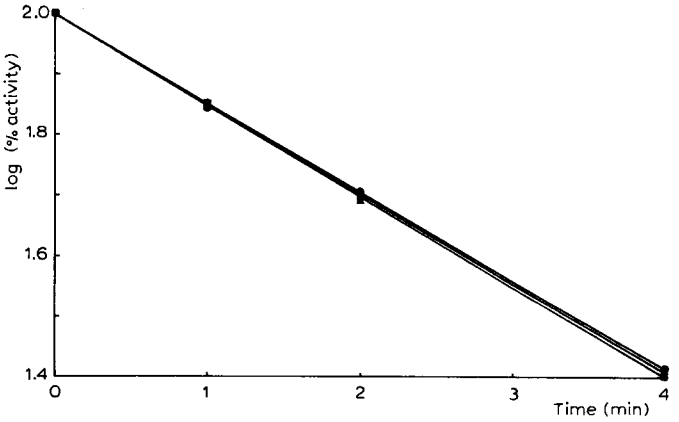

Fig. 5. Effect of pH on the rate of inhibition of creatine kinase by iodoacetamide in the absence of any substrates or anions. ●, pH 7.6; ▲, pH 8.5; ■, pH 9.5. The iodoacetamide concentration was 0.36 mM and the enzyme concentration was 0.57 mg/ml.

TABLE VI

A COMPARISON OF THE ABILITY OF SMALL ANIONS TO INHIBIT CREATINE KINASE ACTIVITY IN THE FORWARD REACTION AND THEIR ABILITY TO PROTECT THE ENZYME FROM IODOACETAMIDE INHIBITION IN THE PRESENCE OF CREATINE PLUS MgADP AT 30°C

For the monkey muscle enzyme inhibition experiments were carried out in the presence of 40 mM creatine. 1 mM MgADP and 10 mM magnesium acetate. The iodoacetamide concentration was 0.36 mM and the enzyme concentration was 0.57 mg/ml. The results for the rabbit muscle enzyme are taken from the work of Milner-White and Watts [5].

Anion (100 mM)	Rabbit muscle enzyme		Monkey muscle enzyme	
	Protection (%)	Inhibition (%)	Protection (%)	Inhibition (%)
Nil	0	0	0	0
NaCl	82	27	66	24
$NaNO_2$	100	81	95	72

The effect of adding products to form the dead-end complex, creatine and MgADP, on the rate of iodoacetamide inhibition over a pH range 7.5—9.5 was examined under various conditions, since Kumudavalli et al. [9] had reported a pH-dependent protection by this complex.

Inhibition of the enzyme by iodoacetamide in the presence of the dead-end complex showed extremely little pH-dependence both at a high enzyme concentration of 0.47 mg/ml (Fig. 4B) and at a low enzyme concentration of 0:0285 mg/ml and no pH dependence when 0.1 M NaCl was also present. The inhibition reaction was also shown to be independent of pH in the absence of any substrates or anions (Fig. 5).

The dead-end complex in conjunction with NaCl or $NaNO_3$ affords the enzyme considerable protection against inactivation by iodoacetamide and these results are compared in Table VI with those obtained for the rabbit muscle enzyme under similar conditions [5].

Discussion

Kinetic studies

The phosphagen kinases function as ATP-regenerating systems and in the course of evolution from a common ancestral arginine kinase the specificity for ATP as principal nucleotide substrate has been maintained while that for the guanidine substrate has evolved to permit a variety of compounds to act as phosphagen formers [18,19]. This difference in the evolution of the two types of substrate-binding site may explain why the dissociation constants for the nucleotide substrates from the free enzyme of different species (assuming rapid equilibrium, random kinetics) tend to span a more limited range than those for the guanidine substrates (Tables I and II). This difference is greatly decreased when the constant for dissociation of the substrate from the enzyme saturated with the second substrate is considered, which presumably reflects the greater restrictions implicit in organising the substrates preparatory to phosphoryl group transfer.

Creatine kinase is generally composed of two subunits, each possessing one catalytic site [15]. No evidence has been found to indicate allosteric

interaction between catalytic sites; however, at least for the mammalian enzymes, synergistic binding of substrates at one catalytic site appears to be a common feature (Tables I and II).

Although from fluorescence measurements an interaction between the two substrates has been observed even in the absence of the enzyme [20], substrate-induced conformational changes of the enzyme-substrate complex are more probably involved in the synergistic substrate-binding effect [15]. The extent of synergism is measured by $K_a/K_{a'}$ ($= K_b/K_{b'}$) for the forward reaction and by $K_p/K_{p'}$ ($= K_q/K_{q'}$) for the back reaction (Tables I and II). For the monkey enzyme the synergistic binding of substrates is greater in the forward direction than in the reverse direction. This, in part, probably reflects the higher pH (8.6 as against 8.0) at which the forward reaction was assayed. Jacobs and Kuby [4] found that for the calf brain enzyme the extent of synergism decreased with pH. Their data show that in going from pH 8.8 to pH 7.4 $K_a/K_{a'}$ falls from 7.85 to 4.3 and $K_b/K_{b'}$ from 7.15 to 3.94 for the forward reaction. For the back reaction $K_p/K_{p'}$ falls from 10 to 1.3 and $K_q/K_{q'}$ from 12 to 1.3. Because the effect of pH is much greater for the back reaction the different natures of the enzyme-substrate complexes must also be important. The present data are in accord with those findings and the suggestion that the conformational organisation of the protein molecule tightens as the pH is lowered.

The monkey muscle enzyme was observed in the present study to be less stable and more prone to oxidation than the enzyme from rabbit muscle. This, and the greater synergism in substrate binding, suggests that it may be a more flexible molecule of "looser" geometric structure. Jacobs and Kuby [4] formed the same conclusions on very similar evidence with regard to the calf brain enzyme and indicated that it may be much more suitable for the study of conformationally dependent processes than the more widely used rabbit muscle enzyme. If the monkey brain enzyme shows the same increase in synergistic binding over the monkey muscle enzyme as the calf brain enzyme shows over the calf muscle enzyme, it could prove to be a very good source of enzyme for the study of such processes.

The fluorescence studies by McLaughlin [21] suggest that a distinguishing feature of the transition-state complex is a substantial interaction between the two subunits. It is of interest to note in this context that preliminary results obtained in this laboratory indicate that the synergism of substrate binding to monkey muscle creatine kinase may well be significantly reduced in the presence of urea.

The "essential" thiol group

Monkey muscle creatine kinase, in common with the enzyme from other sources [15], is readily inactivated by reaction with iodoacetamide. By comparison with the rabbit muscle enzyme, on which detailed investigations have been made, this may be attributed to the alkylation of a single cysteine side chain, although other reactive thiols may be present, as in the dogfish [22] or may become exposed as a result of ageing of the enzyme, as in ox brain [23].

The present work shows that for the monkey enzyme the essential thiol, in the presence of creatine plus MgADP, is not protected against alkylation

until a class II anion is also added (Fig. 4A, Table VI). Furthermore, the rate of alkylation is independent of pH in the presence or absence of the protective substrate complex. Thus the picture is essentially that found with the rabbit enzyme [5,8,25,26].

A previous report by Kumudavalli et al. [9] that alkylation of the essential thiol groups of the enzymes of both human and monkey muscle becomes pH independent in the presence of the anion-stabilised creatine-enzyme-Mg ADP complex was not confirmed. The previous results may possibly be explained by partial oxidation of these rather unstable enzymes (vide supra) causing additional reactive thiols to become exposed as was found with the enzyme from ox brain [23].

Thus there appear to be no exceptions to the generalisation that the essential thiol group becomes less reactive irrespective of pH upon formation of a pseudo-transition-state complex.

Effects of anions

In the absence of a modifying anion, progress curves obtained by the titrimetric assay as described in this work are essentially linear over the measured range. The pronounced curvature of progress curves for the forward reaction in the presence of NO_2^-, NO_3^- or Cl^- is consistent with the formation of a stable, inhibitory enzyme-creatine-anion-MgADP complex as MgADP is formed by the reaction. Also, the degree of curvature increases in the same order as the extent of inhibition caused by different anions. The increase in curvature of the progress curves obtained as the initial MgATP concentration was lowered is also consistent with this theory, since the substrate competitively prevents the binding of MgADP and, consequently formation of the inhibitory complex.

Cl^- and NO_2^- greatly enhance the inhibitory effect of MgADP in the forward reaction (Figs 3B and 3C). The dissociation constants for MgADP and creatine from the enzyme are considerably lowered, but that for MgATP is increased (Table IV). NO_2^- inhibits the forward reaction more than Cl^- (Table VI), has a lower apparent K_i value (Table IV) and causes a greater degree of curvature of the reaction progress curve. NO_2^- also causes a greater reduction of the apparent K_i for MgADP and an increase in the apparent K_m for MgATP (Table V) and, in the presence of creatine and MgADP, affords more protection than Cl^- against iodoacetamide inhibition (Table VI). These results, like those from the iodoacetamide inhibition experiments, are entirely consistent with the formation of a stable, inactive enzyme-creatine-anion-Mg ADP complex as proposed for the rabbit enzyme [5].

Sulphate failed to enhance MgADP inhibition (Fig. 3B) or lower the apparent K_i for MgADP (Table V), but does increase the apparent K_m for MgATP. This evidence is consistent with the anion interacting with the enzyme binding site for the γ-phosphoryl group of the ATP, but being unable, presumably for steric reasons, to mimic the transferable phosphoryl group in a quaternary dead-end-anion complex.

Noda et al. [26] concluded that all anions inhibiting the forward reaction of the rabbit muscle enzyme were competitive with the phosphorylated substrate, but Milner-White and Watts [5] proposed that whilst this was the case

for larger anions such as SO_4^{2-}, small anions, such as Cl^-, were not simply competitive. The conclusions drawn by the latter workers may be questioned as they were based on comparisons of measurements made in the presence of these anions with those made in the presence of acetate, which activates the enzyme. However, the present study confirms, using appropriate controls to avoid the complication of activation by acetate, that inhibition by these anions should still be classified as non-competitive.

Lacombe et al. [27] have disputed the idea that Cl^- inhibits by forming the quaternary complex. They have observed a time-dependent, two-phase inhibition by Cl^-, which is similar to the effect reported for *Sipunculus* arginine kinase [28]. They report that a time-dependent inhibition is still observed with a coupled assay system, which is assumed to remove ADP rapidly and efficiently as it is produced, such as to preclude the possibility of a significant concentration of quaternary complex accumulating. However, the quaternary complex could form if replacement of the phosphocreatine on the enzyme by creatine can occur before dissociation of the MgADP. This matter remains unresolved. Lacombe et al. [27] performed their experiments in the presence of 100 mM acetate, which somewhat confuses the picture since acetate markedly activates the enzyme [5]. In addition, much higher Cl^- concentrations were generally employed, which in the case of the *Sipunculus* enzyme, caused a perturbation of its protein absorption spectrum which could suggest some structural disorganization. Thus the most reasonable explanation of their data is that they were studying a different phenomenon since the type of anion inhibition reported here is now supported by a diversity of findings [5,21,29–32].

Comparison of anion effects on creatine kinase from rabbit muscle and monkey muscle

Although monkey muscle and rabbit muscle creatine kinases react in a similar way to different anions the magnitudes of the effects show considerable species variation. Thus Table III shows that small, planar anions inhibit the monkey muscle enzyme in the same order of effectiveness, but to a slightly different extent than they inhibit the rabbit muscle enzyme [5]. Parallelling these observations, it is clear that Cl^- and NO_2^- not only inhibit the monkey muscle enzyme less, but also in the presence of the dead-end complex, creatine plus MgADP, afford it less protection against iodoacetamide inhibition than the rabbit muscle enzyme (Table VI). Sulphate, on the other hand, which is considered to inhibit only by direct competition with the phosphorylated substrate, inhibits the monkey muscle enzyme much more than it inhibites the rabbit muscle enzyme.

It may be inferred from these results that the fraction of time spent by the monkey muscle enzyme in the enzyme-creatine-anion-MgADP quaternary complex is less than for the rabbit muscle enzyme. This is compatible with the much greater synergistic binding of substrates at one catalytic site found with the monkey creatine kinase. It may also offer a partial explanation of the poor enzyme stability since Jacobs and Cunningham [33] found that the presence of an anion-stabilised dead-end complex, creatine-Cl^--MgADP, (al-

though the nature of the complex was not appreciated by them at the time) enhanced the resistance of the rabbit enzyme to digestion by trypsin.

Acknowledgements

We are indebted to the Muscular Dystrophy Group of Great Britain for financial support and for a personal grant for W.R.C. We would also like to thank the National Institute for Biological Standards and Control, London, N.W.3 for making available a supply of fresh Rhesus monkey muscle. We would like to acknowledge the assistance of Mr. J.A. Underhill during the early part of this work.

References

1 Morrison, J.F. and James, E. (1965) Biochem. J. 97, 37—52
2 Morrison, J.F. and Cleland, W.W. (1966) J. Biol. Chem. 241, 673—683
3 Milner-White, E.J. and Watts, D.C. (1970) Biochem. J. 118, 23P
4 Jacobs, H.K. and Kuby, S.A. (1970) J. Biol. Chem. 245, 3305—3314
5 Milner-White, E.J. and Watts, D.C. (1971) Biochem. J. 122, 727—741
6 Benesch, R.E., Lardy, H.A. and Benesch, R. (1955) J. Biol. Chem. 216, 663—676
7 Watts, D.C. and Rabin, B.R. (1962) Biochem. J. 85, 507—516
8 O'Sullivan, W.J., Diefenbach, H. and Cohn, M. (1966) Biochem. Easton, 5, 2666—2673
9 Kumudavalli, I., Moreland, B.H. and Watts, D.C. (1970) Biochem. J. 117, 513—523
10 Khromov, N.V. and Remisov, A.L. (1953) Zh. Obshch. Khim. 23, 598—605
11 Vogel, A.I. (1956) A Textbook of Practical Organic Chemistry, 3rd edn, Longmans, Green and Co., London
12 Cleland, W.W. (1967) Annu. Rev. Biochem. 36, 77—112
13 Morrison, J.F., O'Sullivan, W.J. and Ogston, A.G. (1961) Biochim. Biophys. Acta 52, 82—96
14 Watts, D.C. and Moreland, B. (1970) Exp. Physiol. Biochem. 3, 1—30
15 Watts, D.C. (1973) The Enzymes, Vol. VIII, 3rd edn, pp. 383—455, Academic Press Inc., New York
16 Focant, B. and Watts, D.C. (1973) Biochem. J. 135, 265—276
17 Florini, J.R. and Vestling, C.S. (1957) Biochim. Biophys. Acta 25, 575—578
18 Morrison, J.F. and Uhr, M.L. (1966) Biochim. Biophys. Acta 122, 57—74
19 Watts, D.C. (1974) Symp. Zool. Soc. London No. 36, 105—127
20 Chegwidden, W.R. (1974) Ph. D. Thesis. University of London
21 McLaughlin, A.C. (1974) J. Biol. Chem. 249, 1445—1452
22 Simonarson, B.A. and Watts, D.C. (1972) Biochem. J. 128, 1241—1253
23 Thompson, A.R., Eveleigh, J.W., Laws, J.F. and Miles, B.J. (1968) Homologous Enzymes and Biochemical Evolution (Thoai, N.V. and Roche, J., eds), pp. 255—277, Gordon and Breach, New York
24 Lui, N.S.J. and Cunningham, L. (1966) Biochem. Easton, 5, 144—149
25 Rabin, B.R. and Watts, D.C. (1960) Nature 188, 1163—1165
26 Noda, L., Nihei, J. and Morales, M.J. (1960) J. Biol. Chem. 235, 2830—2834
27 Lacombe, G., Thiem, N.V. and Thoai, N.V. (1973) Biochim. Biophys. Acta 293, 150—159
28 Thiem, N.V., Lacombe, G. and Thoai, N.V. (1972) Biochim. Biophys. Acta 258, 422—435
29 Reed, G.H., Diefenbach, H. and Cohn, M. (1972) J. Biol. Chem. 247, 3066—3072
30 Reed, G.H. and Cohn, M. (1972) J. Biol. Chem. 247, 3073—3081
31 McLaughlin, A.C., Cohn, M. and Kenyon, G.L. (1972) J. Biol. Chem. 247, 4382—4388
32 Schimerlik, M.I. and Cleland, W.W. (1973) J. Biol. Chem. 248, 8418—8423
33 Jacobs, G. and Cunningham, L.W. (1968) Biochem. Easton, 7, 143—151