STUDIES ON CREATINE PHOSPHOKINASE

by

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INTRODUCTION

Some properties of the enzyme creatine phosphokinase which catalyses the reaction

$$ADP^{**} + PC \rightleftharpoons ATP + Creatine$$

have been described in a recent communication (ENNOR AND ROSENBERG[1]).

Since then several papers have appeared on the subject. Thus KUBY, NODA AND LARDY[2] and NODA, KUBY AND LARDY[3] have described the isolation of the crystalline enzyme from rabbit muscle and some physicochemical properties of the protein, together with evidence of its homogeneity. While the work described below was in progress, two more communications from the same team appeared dealing with kinetic (KUBY *et al.*[4]) and equilibria studies (NODA *et al.*[5]).

Recently OLIVER[6] and CHAPPEL AND PERRY[7] have confirmed ENNOR AND ROSENBERG's[1] finding that AMP cannot accept phosphorus from PC, and that the occurrence of this reaction, as claimed by BANGA[8], was probably due to the presence of myokinase in the system.

The present communication deals with some aspects of the kinetics of the reactions catalysed by creatine phosphokinase, and with some studies on inhibition by sulphydryl reagents.

EXPERIMENTAL

Materials. The materials used were as described in a previous communication (ENNOR AND ROSENBERG[1]), with the exception of the following:

Lewisite. Requisite amounts of lewisite were weighed out and made up to volume with water.

D.A. A stock solution ($1 \cdot 10^{-2}$ *M*) was made up in ethanol, and dilutions were made with water. When this stock solution was diluted 1:10 the solution was slightly opalescent and a precipitate usually appeared after several hours; dilutions were therefore made just prior to use. A solution obtained as a result of higher dilutions remained clear. In tests involving D.A. appropriate controls were set up to check on any inhibition which may have been produced by the ethanol present. Neither lewisite nor D.A. interfered with creatine estimations at the concentrations employed.

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** Abbreviations. The following abbreviations will be used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5'-monophosphate; PC, phosphocreatine; CPK, creatine phosphokinase; TRIS, *tris*(hydroxymethyl)-aminomethane; D.A., diphenylchloroarsine; lewisite, 2-chlorovinyl dichloroarsine; versene, ethylene diamine tetra-acetic acid; CMBA, *p*-chloromercuribenzoic acid.

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ATP and ADP were prepared as described previously (ENNOR AND ROSENBERG[1]), and were further purified as described by COHN[9], using Dowex-1-X 2 anion exchange resin. All samples were homogeneous as judged by paper electrophoresis.

Methods. The assays of enzymic activity were carried out as described earlier (ENNOR AND ROSENBERG[1]), and whenever the substrate concentration was varied specific mention is made in the text. In all cases linear reaction rates were measured.

The reaction was stopped not by the addition of trichloroacetic acid as before but by the addition of 1.0 ml of a mixture containing 20 ml of 0.2 *M* versene, 20 ml of $4 \cdot 10^{-3}$ CMBA and 10 ml of 5 *N* NaOH. This resulted in complete inhibition of enzymic activity and avoided any possibility of PC hydrolysis as may have occurred had the solution been acid. Moreover, even in the case of the reverse reaction where relatively large amounts of protein were present, filtration was not necessary and the amount of protein held in solution did not interfere with the estimation of creatine.

In the initial experiments with Mn^{+2} difficulty was experienced with the measurement of creatine, since in the alkaline medium the Mn^{+2} ion was coloured and rapidly precipitated. This was completely avoided by the addition of a 5 Molar excess of versene to the samples before the addition of the reagents.

Results

Effect of cations on the forward reaction. In the previous communication it was stated that the activity of the enzyme showed an optimum at a definite ratio of Ca^{+2}/enzyme. This view has now been revised on the basis of the present work, in which the enzymic activity was determined in the presence of a range of Ca^{+2} concentrations and at different enzyme levels.

Fig. 1 shows that the observations on the relationship between the initial reaction velocity and Ca^{+2} concentration, at all enzyme levels, conform to a simple Michaelis-Menten relation, *viz.*, to the equation:

$$v = Vx/Kx + x$$

where v = initial velocity at a standard concentration of substrate and ADP,
V = maximum velocity,
x = Ca^{+2} concentration,
Kx = dissociation constant of the enzyme-Ca^{+2} complex formed according to the equation enzyme + $Ca^{+2} \rightleftharpoons$ enzyme-Ca^{+2}.

This result may be interpreted as indicating that CPK reacts with Ca^{+2} in the ratio of one Ca^{+2} ion for each active centre of the enzyme. Thus the Ca^{+2}-enzyme complex may be considered as an active form of CPK.

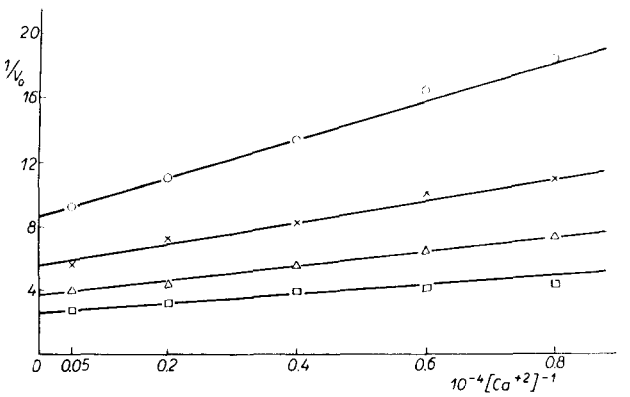

Fig. 1. Dissociation constant for Ca^{+2}. Conditions: 0.05 *M* Tris buffer, pH 7.2, PC and ADP, $2.5 \cdot 10^{-4}$ *M*. 2 minutes at 37°; Ca^{+2} concentration as shown, and CPK content as follows: O——O 5 μg lyophilised enzyme/ml; ×——× 7.5 μg lyophilised enzyme/ml; △——△ 10.0 μg lyophilised enzyme/ml; □——□ 15.0 μg lyophilised enzyme/ml. *Vo* in arbitrary units.

TABLE I

Dissociation constant for Ca^{+2}.
Incubation at 37°, initial concentration of both ADP and PC $2.5 \cdot 10^{-4}$ *M*.

Experiment	[*CPK*], μg protein/ml	"*Km*"
1	5.0	$1.3 \cdot 10^{-4}$
	7.5	$1.3 \cdot 10^{-4}$
	10.0	$1.3 \cdot 10^{-4}$
	15.0	$1.2 \cdot 10^{-4}$
2	7.5	$1.4 \cdot 10^{-4}$
	15.0	$1.3 \cdot 10^{-4}$

Table I shows the calculated values for the dissociation con-

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stant at four enzyme levels. The dissociation constant represents the amount of Ca^{+2} required to form half the maximum concentration of active enzyme complex. The fact that the same dissociation constant is found, irrespective of the amount of enzyme present, indicates that the absolute Ca^{+2} concentration is of importance rather than the Ca^{+2} enzyme ratio.

It was found previously (ENNOR AND ROSENBERG[1]) that no difference could be detected between the activating effect of Ca^{+2} and Mg^{+2} on the reaction. This finding has now been confirmed on the basis of the dissociation constants and maximum velocities. The dissociation constants for both Ca^{+2} and Mg^{+2} were determined simultaneously under identical conditions using stock solutions containing PC, TRIS and CPK for each metal. The resultant data were too close to permit plots on one figure, and are shown in Figs. 2 and 3. As seen from the figures the values of Vmax. at corresponding enzyme concentrations are not significantly different and similar values were obtained for the dissociation constants (Ca^{+2} and Mg^{+2}–$1.3 \cdot 10^{-4}$ M, $2 \cdot 10^{-4}$ M respectively).

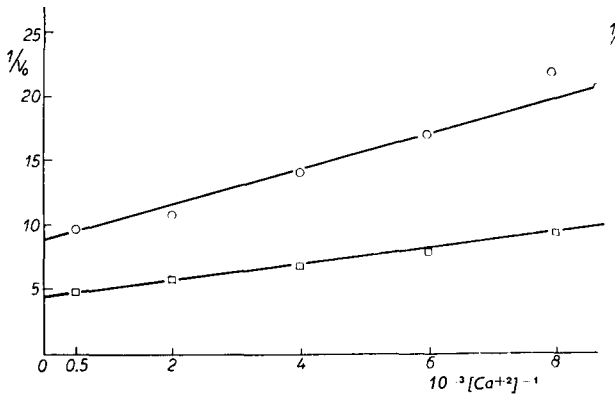

Fig. 2. Dissociation constant for Ca^{+2}. For incubation conditions see Fig. 1. Enzyme concentration: O——O 5 μg lyophilised CPK/ml; □——□ 10 μg lyophilised CPK/ml.

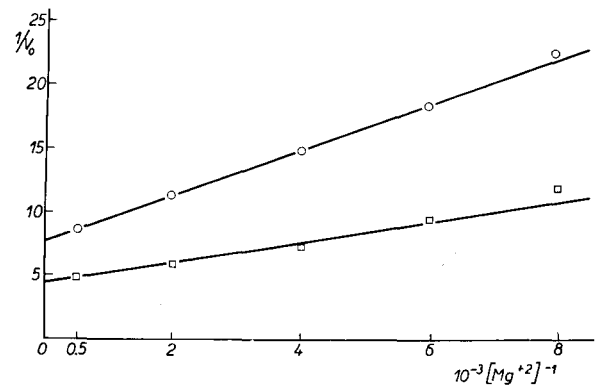

Fig. 3. Dissociation constant for Mg^{+2}. Substrate and enzyme concentration as in Fig. 2.

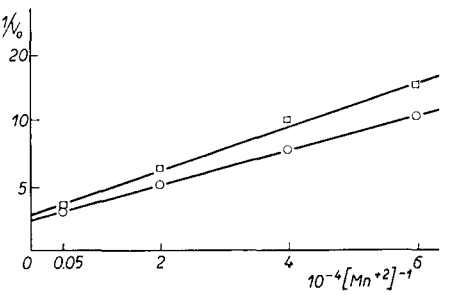

Fig. 4. Dissociation constant for Mn^{+2}. For conditions see Fig. 1. Enzyme concentrations: □——□ 7.5 μg CPK/ml; O——O 10.0 μg CPK/ml.

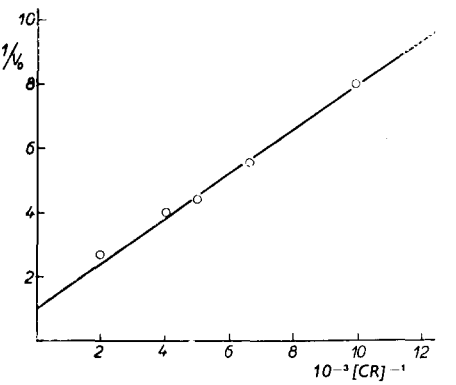

Fig. 5. Michaelis constant for creatine. 0.05 M TRIS buffer, pH 10.2, ATP $2.5 \cdot 10^{-4}$ M. Molar ratio, Ca^{+2}/ATP = 0.4 throughout. Enzyme concentration: 125 μg CPK/ml. Incubation: 2 min, 30°.

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Effect of Mn^{+2}. Mn^{+2} was found to activate the forward reaction giving high initial velocities. As shown in Fig. 4, when the reciprocals of the initial reaction velocities are plotted against the reciprocals of the Mn^{+2} concentrations according to the method of LINEWEAVER AND BURK[10], a straight line is obtained. This indicates that a Michaelis-Menten relationship exists between Mn^{+2} concentration and CPK activity. Thus Mn^{+2} can replace Ca^{+2} and Mg^{+2} to form an active enzyme complex.

The dissociation constant calculated from the plot is $6 \cdot 10^{-5}$ *M* (mean value) and is significantly lower than the corresponding constants for Mg^{+2} and Ca^{+2}.

Figs. 2, 3 and 4 are plotted on the same scale, and the lower line in each plot corresponds to the same enzyme concentration in each case (10 μg/ml). From these figures the relative maximum initial velocities obtained with each metal may be calculated; these are in the ratio 1 : 1 : 2 for Ca^{+2}, Mg^{+2} and Mn^{+2} respectively.

Effect of substrate concentration on initial velocity. The effect of increasing concentrations of creatine and ATP in the reverse reaction and of phosphocreatine in the forward reaction is shown in Figs. 5, 6 and 7 respectively. A Michaelis-Menten relation was obtained with these substrates.

The K_m value of CPK for creatine was found to be $7.0 \cdot 10^{-1}$ *M* and for PC, $2 \cdot 10^{-2}$ *M*.

In the case of ATP the LINEWEAVER-BURK[10] plot did not give a straight line when the concentration of ATP was varied and the concentration of Ca^{+2} held constant. This was presumably due to the co-ordination effect of ATP, but as seen from Fig. 8 maximum activity under the experimental conditions was obtained when the ATP/Ca^{+2} ratio was about 2.5.

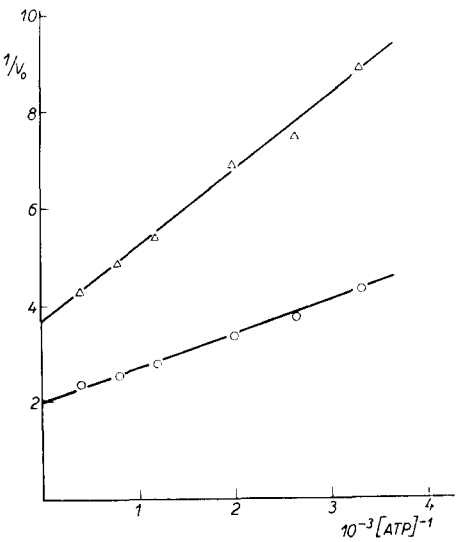

Fig. 6. Michaelis constant for ATP. 0.05 *M* TRIS buffer, pH 10.2. Creatine, $2.5 \cdot 10^{-4}$ *M*. Molar ratio, Ca^{+2}/ATP = 0.4 throughout. Enzyme concentration: 125 μg CPK/ml. Incubation: 2 minutes, 30°.

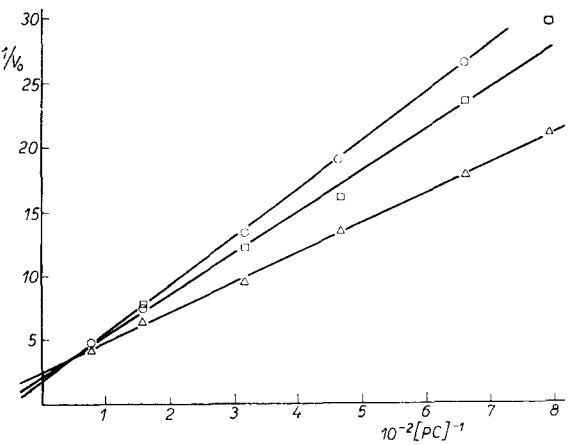

Fig. 7. Michaelis constant for PC. 0.05 *M* TRIS, pH 7.2; Ca^{+2}, $5 \cdot 10^{-4}$ *M*; ADP, $2.5 \cdot 10^{-4}$ *M*. Incubation: 2 minutes, 37°.

Using ATP and Ca^{+2} at this ratio in subsequent reactions, it was possible to obtain a straight line plot (Fig. 6 above), and the K_m value of CPK for ATP was calculated to be $3.3 \cdot 10^{-4}$ *M*.

In our system the effect of ADP differed from that of other substrates in that, with concentrations of 10^{-3}–10^{-4} *M*, ADP showed a marked inhibition of the initial

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velocity. At first this was thought to be due to co-ordination of the Ca^{+2} necessary for enzyme action. The inhibition, however, persisted when this possibility was eliminated.

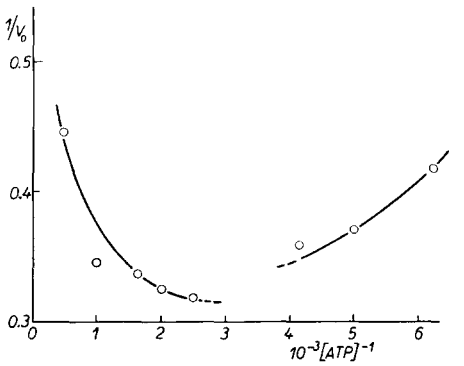

Fig. 8. Effect of ATP on the reverse reaction in the presence of a constant amount of Ca^{+2} ($1 \cdot 10^{-4}$ *M*). 0.05 *M* TRIS buffer, pH 10.2, creatine $2.5 \cdot 10^{-4}$ *M*. Enzyme concentration: 125 μg CPK/ml.

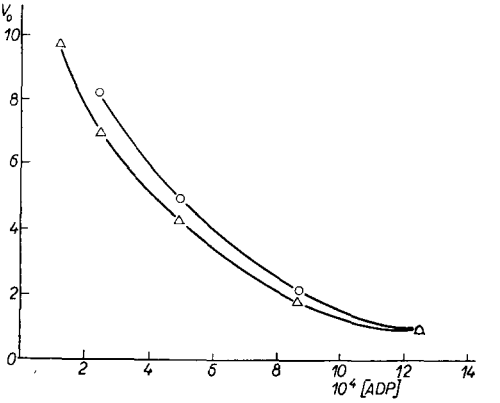

Fig. 9. Effect of ADP on CPK activity. Forward reaction: 0.05 *M* TRIS, pH 7.2, $1.25 \cdot 10^{-3}$ *M* PC. CPK, 4 μg/ml. Incubation: 2 min., 30°. △——△ Constant $[Ca^{+2}] = 5 \cdot 10^{-4}$ *M*. O——O Constant $[Ca^{+2}]/[ADP] = 4.0$.

Fig. 9 shows the effect of ADP concentration on the initial velocity both at constant Ca^{+2} concentration and at constant Ca^{+2}/ADP concentration ratio. The experiment was repeated several times with different batches of ADP in order to eliminate the possibility of an extraneous inhibitor, but in every case the pattern was similar to that shown in Fig. 9.

A decrease in the reaction velocity with decreasing concentration of ADP was finally obtained at considerably lower concentrations of ADP and with a Ca^{+2}/ADP ratio of 4:1. A plot of the inverse of the reaction velocity against the inverse of the ADP concentration failed, however, to show a Michaelis-Menten relation because apparently at the lower ADP concentrations initial velocities were no longer measured. It was not practicable, however, to test this by using lower enzyme concentrations or shorter incubation times, for both procedures would have resulted in the release of amounts of creatine too small to permit accurate estimation.

Effect of sulphydryl reagents

Lewisite. It was found that lewisite produced no inhibition in concentrations up to $2.5 \cdot 10^{-4}$ *M*.

Diphenylchloroarsine. The inhibitory effect of varying concentrations of D.A. and the complete reversal of the inhibition by cysteine is shown in Table II.

DISCUSSION

There is some disagreement between the present data and published work. Thus the dissociation constant for Ca^{+2} and Mg^{+2} in the forward reaction were 1.3 and $2 \cdot 10^{-4}$ *M* respectively, whilst KUBY *et al.*[4] found a value of $2 \cdot 10^{-3}$ *M* for Mg^{+2}. The value for Ca^{+2} was not quoted by these authors. More marked divergence between the results is found

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TABLE II

THE EFFECT OF LEWISITE AND DIPHENYLCHLOROARSINE (D.A.) ON CPK ACTIVITY, AND THE REVERSAL OF THE INHIBITION BY CYSTEINE

Inhibitor	*Concentration (M) of inhibitor*	*% Inhibition*
Lewisite	$2.5 \cdot 10^{-4}$	0
D.A.	$2.0 \cdot 10^{-5}$	21
D.A.	$1.5 \cdot 10^{-4}$	66
D.A.	$2.5 \cdot 10^{-4}$	82
D.A. + cysteine $1.25 \cdot 10^{-3} M$	$2.5 \cdot 10^{-4}$	0

with Mn^{+2}, which in the present work gave a dissociation constant of $6 \cdot 10^{-5} M$, whereas the value found by KUBY *et al.* was $2 \cdot 10^{-3} M$. The data on relative maximal velocities are also in disagreement. Unlike KUBY *et al.*[1] who found that Ca^{+2} would give only one-half the initial velocity which was obtained with Mg^{+2}, we have not found any significant difference between these metal activators.

These discrepancies are too great to be accounted for by experimental error, particularly since the LINEWEAVER AND BURK[10] plots, in all cases, are plotted on a significant number of points. It would seem likely that the explanation lies in the nature of the buffering systems employed in the experiments. Of the systems tested KUBY *et al.*[1] found that TRIS gave the lowest reaction velocity, and NODA *et al.*[5] have expressed the opinion that interaction between buffer and metal activator might affect the calculated values of dissociation constants. The large differences between the values quoted in the present communication (using TRIS) and those of KUBY *et al.*[1] (using glycylglycine) might then be due to such interaction.

Because of the fact that the CPK used in the present experiments was not a pure protein, it is not permissible to compare the absolute initial velocities with those obtained by KUBY *et al.*[1].

The high initial velocities, together with the apparently greater affinity of the enzyme for Mn^{+2}, suggest that this metal might be the true "*in vivo*" activator of CPK.

The experimental results obtained with the arsenicals, lewisite and D.A., are of interest for they give some indication of the proximity of the –SH groups on the enzyme molecule. CPK is not sensitive to the action of lewisite, and it may thus be inferred that the –SH groups which are known to be essential for enzyme action (ENNOR AND ROSENBERG[1]) cannot form a stable >As-ring (*cf.* LOTSPEICH AND PETERS[11]). D.A., on the other hand, proved to be an effective inhibitor, presumably giving a stable monothioarsenite, and the inhibition could be quantitatively reversed by the addition of cysteine in a molar ratio of 5:1. On the basis then of the work of the Oxford school (PETERS[12]) it may be presumed that this is a monothiol attack.

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SUMMARY

1. Some aspects of the kinetics of the CPK catalysed reaction PC + ADP $\rightleftharpoons$ ATP + creatine and the effects of certain arsenical inhibitors have been studied.

2. CPK is activated by Ca^{+2}, Mg^{+2} and Mn^{+2}, and the reaction velocity is greatest in the presence of the latter ion.

3. The effects of lewisite and diphenylchloroarsine suggest that CPK is a "monothiol" enzyme.

RÉSUMÉ

1. Quelques aspects cinétiques de la réaction catalysée par la CPK, PC + ADP $\rightleftharpoons$ ATP + créatine, et les effets de certains inhibiteurs arsénicaux ont été étudiés.

2. La CPK est activée par Ca^{+2}, Mg^{+2} et Mn^{+2}, et la vitesse de la réaction est plus grande en présence de ce dernier ion.

3. Les effets de la lewisite et de la diphénylchloroarsine suggèrent que la CPK est un enzyme "monothiol".

ZUSAMMENFASSUNG

1. Einige, mit der Kinetik der durch CPK katalysierten Reaktion PC + ADP $\rightleftharpoons$ ATP + Kreatin in Verbindung stehende Fragen, sowie die Wirkung gewisser hemmenden Arsenverbindungen wurden erforscht.

2. CPK wird durch Ca^{+2}, Mg^{+2} und Mn^{+2} Ionen aktiviert und die Reaktionsgeschwindigkeit ist in Gegenwart des letztgenannten Ions am grössten.

3. Die Wirkung von Lewisit und Diphenylchloroarsin lässt vermuten, dass es sich um ein "Monothiolenzym" handelt.

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