INHIBITION OF CREATINE KINASE BY CREATININE PHOSPHATE

G. GERCKEN and V. DÖRING
Institut für Organische Chemie und Biochemie, Abt. für Biochemie, Universität Hamburg, Hamburg, Germany

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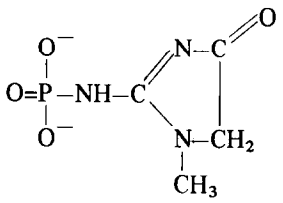
1. Introduction

The mechanism of the creatine kinase reaction has been shown to be random order, rapid equilibrium [1]. The specificity of creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) in relation to magnesium–nucleotide complexes was studied by James and Morrison [2] and the specificity in relation to guanidino substrates by McLaughlin and Cohn [3]. Clark and Warren [4] postulated that creatinine phosphate, the cyclic anhydride of creatine phosphate (CP), could function as phosphoryl group donor in the creatine kinase reaction. Creatinine phosphate was identified by Zeile and Meyer [5] showing that the phosphoryl residue is bound at the exocyclic nitrogen atom. Spectroscopic data gave evidence for the double bond being located in the five-membered ring system of glycocyamidines [6–8].



Creatinine phosphate

Broun [9,10] has described the reaction of creatinine phosphate with ADP catalysed by creatine kinase and mentioned that the compound would be suitable to function as donor of an energy-rich phosphate residue in cell metabolism. We investigated the properties of creatinine phosphate as substrate and inhibitor for creatine kinase and alkaline phosphatase (EC 3.1.3.1).

2. Methods and materials

The kinetic of the reverse reaction of creatine kinase was determined by an optical enzyme assay with the aid of the hexokinase and glucose 6-phosphate dehydrogenase reaction. In the auxiliary system the concentrations of the components for the coupled reactions were adjusted so that the auxiliary system did not limit the rate of the overall reaction. In a final volume of 3.5 ml the assay medium contained 0.05 M triethanolamine buffer, pH 7.05; 3 mM D-glucose monohydrate; 2.58 mM $MgCl_2 \cdot 6\ H_2O$; 0.154 mM NADP; 0.5 mM ADP trisodium salt (varied CP concentration) or 0.05–0.5 mM ADP trisodium salt (constant CP concentration); 11.44 mM CP disodium salt $\cdot 6\ H_2O$ (varied ADP concentration) or 1.43–17.16 mM CP disodium salt $\cdot 6\ H_2O$ (constant ADP concentration); 5.72 or 11.44 mM creatinine phosphate disodium salt $\cdot 5\ H_2O$ (inhibition experiments); 20 μg of hexokinase (about 2.8 U); 15 μg of glucose 6-phosphate dehydrogenase (about 2.1 U); 62.5 ng of creatine kinase (about $5.0 \cdot 10^{-3}$ U). The reaction was started by addition of creatine kinase and the change in absorbance at 334 nm was followed in intervals of 30 sec (d = 4 cm, T = 30°C).

In order to investigate enzymatic hydrolysis 12.5 μmoles of creatinine phosphate were incubated with 0.1 mg of creatine kinase (0.05 M triethanolamine buffer, pH 7.05, T = 30°C) or with 20–80 μg of

alkaline phosphatase (0.1 M glycine/NaOH buffer, pH 9.4, $T = 20°C$) for 30–120 min. Inorganic phosphate was determined by the method of Berenblum and Chain [11] modified by Martin and Doty [12].

After measuring the kinetics of highly purified creatine kinase of rabbit skeletal muscle (Boehringer Mannheim, Mannheim, Germany) we investigated the inhibition of creatine kinase activity in rabbit heart homogenates with regard to the possibility of an in vivo inhibition.

Rabbit hearts were homogenized in 15 ml of 0.05 M triethanolamine buffer, pH 7.05, by an Ultra-Turrax homogeniser. The homogenate was centrifuged at 15 000 *g* for 30 min, in order to remove NADPH dehydrogenase. The supernatant was diluted 1:1000. 0.02 ml of the diluted supernatant were used for each assay. For protein determination the method described by Lowry et al. [13] was used with bovine serum albumine as standard.

Enzymes and coenzymes were purchased from Boehringer Mannheim, Mannheim, Germany. Creatinine phosphate was a gift from Sanol-Arzneimittel Dr Schwarz GmbH, Monheim, Germany.

3. Results and discussion

In assays with creatinine phosphate as substrate for creatine kinase no reaction was observed. Therefore, it is obvious that creatinine phosphate cannot be used by creatine kinase as donor of the phosphoryl group in order to accomplish ATP formation. The hypothesis of Clark and Warren [4] stating that in the forward reaction creatine and in the reverse reaction creatinine phosphate serve as substrates could not be verified. Furthermore, this result put in question the in vivo utilisation of creatinine phosphate as donor of an energy-rich phosphate group [9,10].

The rate of spontaneous hydrolysis of creatinine phosphate was not accelerated by the addition of creatine kinase at 30°C and pH 7.0 indicating that there was no phosphatase activity in the creatine kinase preparation in relation to creatinine phosphate.

The kinetics of the creatine kinase reaction were determined as a function of the creatine phosphate concentration when the ADP concentration was held constant at 0.5 mM. In fig. 1 the experimental data of v_0 have been plotted in the double reciprocal

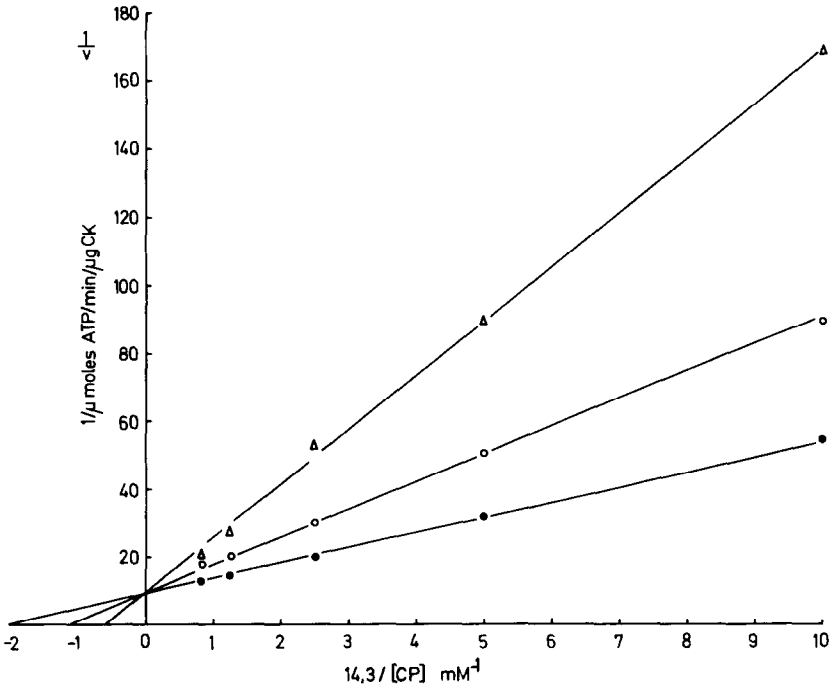


Fig. 1. Competitive inhibition of the initial velocity of the creatine kinase reaction by creatinine phosphate with creatine phosphate as variable substrate. pH 7.05, T = 30°C, ADP concentration 0.5 mM. (●) no added creatinine phosphate, (○) 5.72 mM, (△) 11.44 mM creatinine phosphate. (Means of three experiments.)

Lineweaver–Burk diagram. Addition of creatinine phosphate in two concentrations effected a competitive inhibition. When the ADP concentration was varied and the CP concentration held constant the kinetic data showed a noncompetitive inhibition by creatinine phosphate (fig. 2). A replot of the intercepts from Lineweaver–Burk diagram (fig. 1) versus the inhibitor concentration resulted in an apparent $K_i = 5 \cdot 10^{-3}$ (fig. 3). As creatinine phosphate acts as competitive inhibitor with respect to CP and as noncompetitive inhibitor with respect to ADP it can be assumed that creatinine phosphate as an analog of CP competes with CP for the binding site at the enzyme not influencing the binding of ADP. A conversion of the central enzyme–MgADP–creatinine phosphate complex to an enzyme–MgATP–creatinine complex is, however, not possible. Accordingly, creatinine is not phosphorylated in the forward reaction. The difference between creatine and creatinine or their *N*-phosphorylated derivatives, respectively, depends on the intact or lactonized carboxyl function. It can be concluded that the carboxyl group is significant for the steric orientation and activation of the guanidino compound at the active site of creatine kinase. In experiments of McLaughlin and Cohn [3] 1-carboxy-

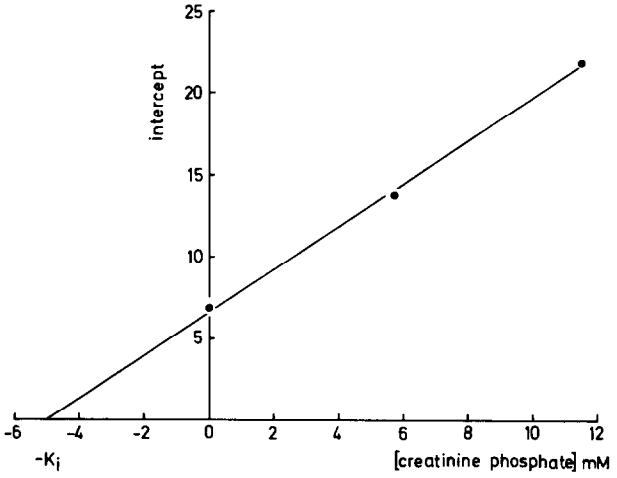


Fig. 3. Replot of intercepts from fig. 1 versus creatinine phosphate concentration.

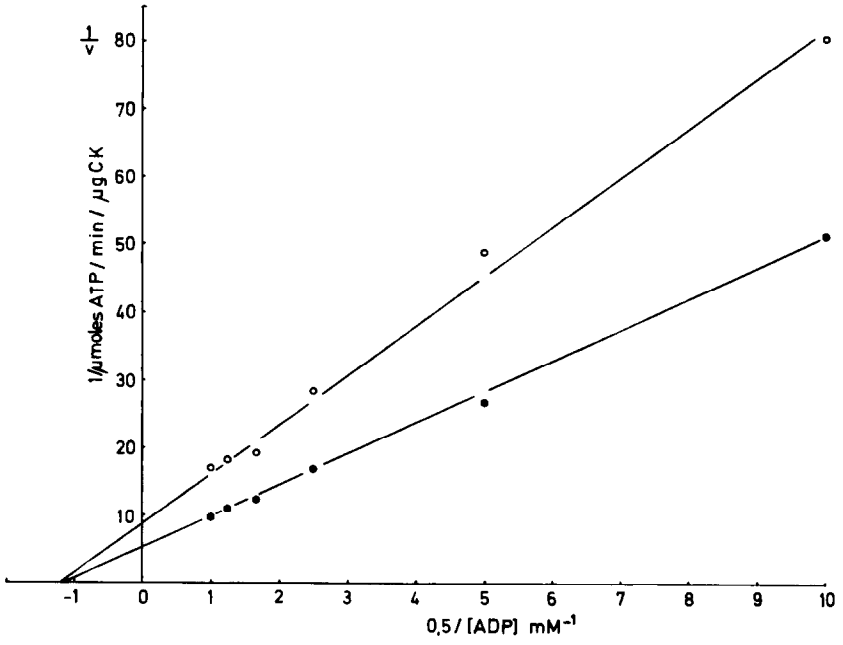


Fig. 2. Noncompetitive inhibition of the initial velocity of the creatine kinase reaction by creatinine phosphate with ADP as variable substrate. pH 7.05, $T = 30°C$, creatine phosphate concentration 11.44 mM. (•) no added creatinine phosphate, (○) 5.72 creatinine phosphate. (Means of three experiments.)

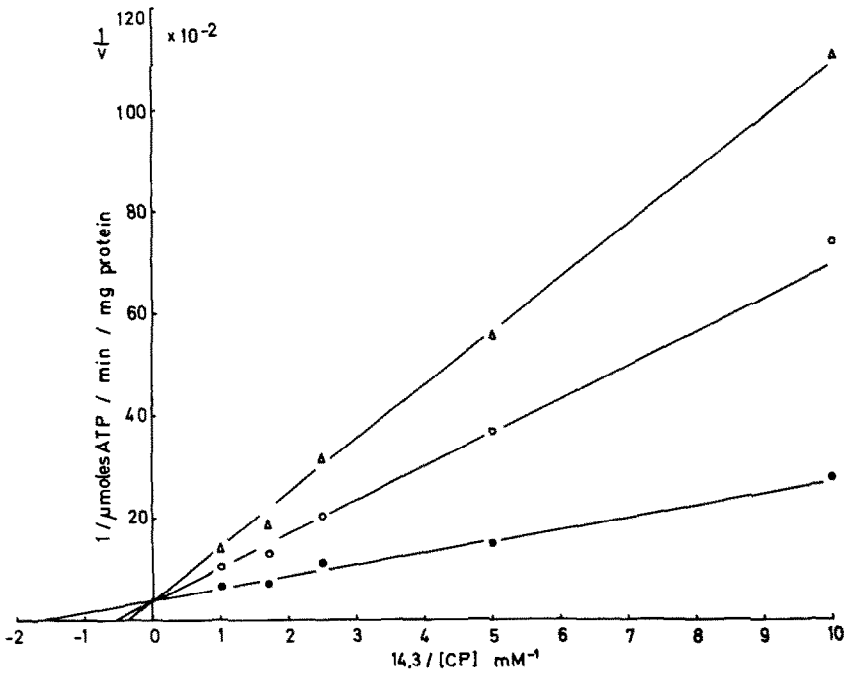


Fig. 4. Competitive inhibition of the initial velocity of the creatine kinase activity in heart muscle homogenate of rabbit with creatine phosphate as variable substrate. pH 7.05, $T = 30°C$, ADP concentration 0.5 mM. (●) no added creatinine phosphate, (○) 5.72 mM, (△) 11.44 mM creatinine phosphate. (Means of three experiments.)

methyl-2-iminoimidazolidine was the most reactive analog of creatine in the forward reaction. This is in accordance with the expectation that the five-membered ring analog inhibited the reverse reaction. Although the kinetics of the inhibition was of competitive type in relation to CP the inhibition has to be understood as product inhibition. Inverse to the inhibition type of creatinine phosphate the magnesium-nucleotide complexes MgCDP, MgUDP and MgGDP caused competitive inhibition with respect to MgADP and noncompetitive inhibition with respect to CP [2].

The results found with highly purified creatine kinase of skeletal muscle were proved by those obtained with creatine kinase activity of rabbit heart homogenates. The creatine kinase activity in the homogenates was also competitively inhibited by creatinine phosphate (fig. 4). In experiments with heartlung preparations of the rabbit the addition of creatinine phosphate suggests an in vivo inhibition of creatine kinase [14]. The hearts became dilated and insufficient. Metabolite determinations showed a diminished ATP content and a normal or even increased CP content. Until now in investigations of muscle metabolism the unspecific sulfhydryl reagent 1-fluoro-2,4 dinitrobenzene was used for inhibition of creatine kinase [15–17]. In further investigations the use of creatinine phosphate can be very helpful because of its great specificity for this essential reaction in muscle metabolism.

4. Summary

Creatinine phosphate was shown to be no substrate of purified creatine kinase from skeletal muscle or of creatine kinase in heart muscle homogenate. It was found to be a competitive inhibitor with respect to creatine phosphate and a noncompetitive inhibitor with respect to ADP.

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