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A Quenched-Flow Study of the Reaction Catalysed by Creatine Kinase

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The reaction catalysed by creatine kinase was studied in both directions by quenched-flow techniques to follow the initial product formation in the millisecond range. In both directions the amount of product formed increases linearly with time, and the turnover number corresponds to the steady-state value. Extrapolation to zero time indicates the absence of either a large transient phase or a large lag phase in both directions. This indicates that the actual chemical reaction is rate-limiting, and that all possible isomerizations before or after the chemical step must be much more rapid.

The synthesis of creatine phosphate:

$$MgATP^{2-} + Cr \rightleftarrows MgADP^{-} + CrP^{2-} + H^{+}$$

catalysed by the enzyme creatine kinase (EC 2.7.3.2) has been studied extensively by steady-state kinetic methods. The results obtained point to a rapid-equilibrium random mechanism (Watts, 1973, and references therein) with the metal nucleotides as true substrates. Isotope-exchange studies (Morrison & Cleland, 1966) also showed the interconversion of substrates into products to be rate-limiting. This process may, however, still include isomerization steps next to the chemical reaction. There is indeed strong evidence, from immunological experiments, chemical modifications and spectroscopic studies, that conformational changes accompany or follow the formation of the ternary complex (Watts, 1973, and references therein).

Relaxation studies with pH indicators (Hammes & Hurst, 1969) allowed the determination of the rate constants associated with the initial association–dissociation of the metal nucleotides and with some subsequent isomerization steps. These were found to be more rapid than the turnover of the substrates.

In the present study quenched-flow techniques have been used to follow the initial product formation on the enzyme. If a rate-limiting isomerization would occur before the chemical reaction, a lag phase would be found in the initial product formation (Gutfreund, 1972). If, however, a rate-limiting step would occur after the chemical reaction, but before product release, a transient phase of product formation on the enzyme would be found (Gutfreund, 1972).

Quenched-flow techniques allow the localization of the rate-limiting step relative to the step of product formation.

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Materials and Methods

Creatine kinase was prepared from rabbit muscle by procedure B of Kuby *et al.* (1954). Fraction IV was dialysed against 10mM-sodium glycinate buffer, pH9.0, and freeze-dried. The enzyme had a specific activity of 150 (±5) μmol·min^{-1}·mg^{-1} (of protein) when assayed at 37°C and pH8.0 for proton release in the pH-stat. A test solution of the following composition was used: 40mM-creatine, 4mM-ATP, 5mM-magnesium acetate, 5mM-sodium acetate and 1mM-dithiothreitol. Protein concentration was determined spectrophotometrically at 280nm by using $E_{1\%} = 8.8\,cm^{-1}$. A molecular weight of 81000 (Kuby *et al.*, 1954) was used in the calculations. The specific activity is about 63% of the maximal value found (Watts, 1973) for crystallized enzyme.

For the forward reaction [γ-^{32}P]ATP was used as the radioactive substrate. It was prepared by the method of Glynn & Chappell (1964) from $^{32}P_i$ obtained from The Radiochemical Centre (Amersham Bucks., U.K.). In view of the instability of creatine phosphate, [β-^{32}P]ADP was preferred as the radioactive substrate in the reverse reaction. It was obtained from [γ-^{32}P]ATP and AMP with myokinase. Both radioactive nucleotides were purified on Whatman DE-52 DEAE-cellulose by using a gradient from 0 to 0.5M-KCl in 20mM-Tris–acetate buffer, pH8.0. Nucleotide concentrations were determined spectrophotometrically from $\varepsilon = 1.54 \times 10^4\,M^{-1}\cdot cm^{-1}$ at 259nm. The contamination of [γ-^{32}P]ATP with $^{32}P_i$ and of [β-^{32}P]ADP with [γ-^{32}P]ATP was lower than 5%.

The quenched-flow apparatus was described by Gutfreund (1969). The general procedure was as follows: 1.5ml of enzyme solution, containing creatine or creatine phosphate concentrations as specified in the Results section, was mixed in the quenched-flow apparatus with an equal volume of radioactive nucleotide solution and ejected in a

glass vial through capillary tubes of different lengths. The glass vial contained 2ml of quenching solution consisting of 20% (w/v) $HClO_4$ and 20mM-EDTA. Immediately after quenching, 1ml of a solution of 4M-sodium acetate–0.1M-KH_2PO_4 was added to raise the pH to 3.4, and the protein precipitate was filtered off by passing the solution through a Whatman glass-fibre paper filter GF/D fitted at the bottom of a plastic syringe. The clear solution was immediately frozen in an acetone–solid CO_2 mixture. For analysis the samples were thawed and 20μl portions were applied to a polyethyleneimine-cellulose thin-layer plate and developed in 0.75M-KH_2PO_4 adjusted to pH3.4 with HCl. The radioactive bands were cut out and eluted with 0.5ml of 1M-HCl. After 15min 15ml of scintillation fluid (made from 3 litres of toluene and 30g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (Koch–Light, Colnbrook, Bucks., U.K.) were added, and radioactivity was counted in a Nuclear–Chicago Unilux II counter. Corrections for background were applied and the labelled ATP or creatine phosphate formed was expressed relative to the total radioactive isotope content for each assay. The specific radioactivity was always about 4×10^{11}c.p.m./mol.

Zero-time points were obtained by mixing the protein with the quenching solution before the addition of the labelled ATP or ADP. Radioactive creatine phosphate runs in the same place as P_i, so that its acid hydrolysis, occurring after mixing with the quenching solution, has no influence on the measurements. ATP and ADP are much less acid-labile. Their hydrolysis is minimized by the procedure explained above.

Results and Discussion

In the direction of creatine phosphate synthesis the formation of the radioactive product (and the disappearance of [γ-^{32}P]ATP) was measured. The results of two experiments, carried out at room temperature (22°C), are shown in Fig. 1. For the first experiment (○) the mixing-chamber concentrations of enzyme and substrates were as follows: 60μM-creatine kinase (the concentration of creatine kinase refers to site concentration), 1.8mM-ATP, 20mM-creatine, 10mM-magnesium acetate, 10mM-sodium acetate and 1mM-dithiothreitol, in 50mM-Tris–acetate buffer, pH8.0. Although this high concentration of Mg^{2+} is slightly inhibitory, it is preferred to give a complete conversion of ATP into MgATP. In the second experiment (●), the conditions were the same except for 134μM-creatine kinase and 2.3mM-ATP. The enzyme saturation can be estimated by using the overall dissociation constant of 7.4mM[2] (Morrison & James, 1965), and was 83 and 86% respectively in the two experiments.

As is clear from Fig. 1, the production of radioactive creatine phosphate is linear with time. The slope of the line corresponds to a turnover number of $33s^{-1}$ (○) and $41s^{-1}$ (●) respectively. When the enzyme was assayed in the pH-stat under the same conditions an average specific activity of 60μmol·min^{-1}·mg^{-1} (of protein) was found, equivalent to a turnover number of $40s^{-1}$. The values agree very well. The least-squares procedure gives correlation coefficients of 0.97 for both, and standard deviations of the ordinate of 5 and 11μM respectively. The concentration present at zero time corresponds to the contamination of the original substrate. Although, owing to the limited accuracy of the results, a small transient or lag phase cannot be excluded, the results indicate that there is no rate-limiting step between product formation and product release. This is in sharp contrast with a number of enzymes, where a rate-limiting isomerization occurs before product release. This is the case with lactate dehydrogenase (Holbrook & Gutfreund, 1973) and myosin (Bagshaw & Trentham, 1973).

In the direction of ATP synthesis, the formation of [β-^{32}P]ATP (and disappearance of [β-^{32}P]ADP) was measured (Fig. 2). The concentrations of enzyme and substrates in the mixing chamber were as follows: for the first experiment (○) 73μM-creatine kinase, 1.3mM-ADP, 10mM-creatine phosphate, 10mM-magnesium

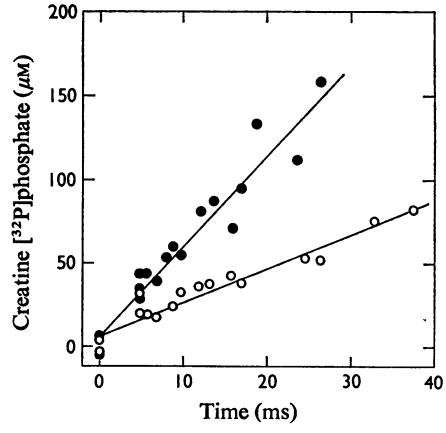

Fig. 1. *Initial formation of radioactive creatine phosphate after mixing in the quenched-flow apparatus*

○, The mixing-chamber concentrations were 60μM-creatine kinase sites, 1.8mM-ATP, 20mM-creatine, 10mM-magnesium acetate, 10mM-sodium acetate and 1mM-dithiothreitol in 50mM-Tris–acetate buffer, pH8.0. ●, The concentrations were the same except for 134μM-creatine kinase sites and 2.3mM-ATP. The origin of the ordinate is taken at the average amount of radioactivity due to contamination with radioactive phosphate.

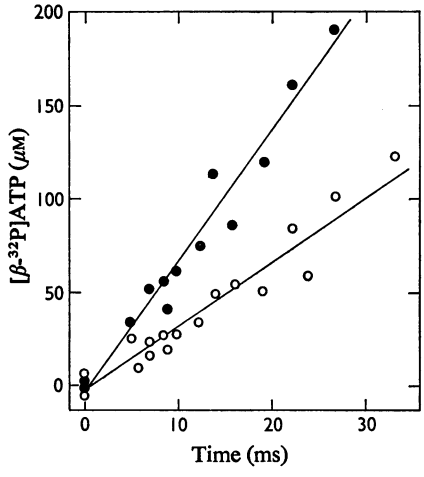

Fig. 2. *Initial formation of radioactive ATP after mixing in the quenched-flow apparatus*

○, The mixing-chamber concentrations were 73 μM-creatine kinase sites, 1.3 mM-ADP and 10 mM-creatine phosphate. The same buffer system was used as in Fig. 1. The second experiment (●) was designed as a pulse–chase experiment (see the text). The average amount of radioactivity due to contamination with radioactive ATP is taken as the origin of the ordinate.

acetate, 10 mM-sodium acetate, 1 mM-dithiothreitol, in 50 mM-Tris–acetate buffer, pH 8.0. From the overall dissociation constant of 0.46 mM[2] (Morrison & James, 1965) the degree of saturation is estimated at 96%. A straight line was obtained, giving a turnover number of $48\,s^{-1}$ (correlation coefficient = 0.98 and standard deviation of $y = 9\,\mu M$). In the pH-stat assay a specific activity of $72\,\mu mol \cdot min^{-1} \cdot mg^{-1}$ was measured, which is equivalent to $48\,s^{-1}$. The second experiment was designed as a pulse–chase experiment. Here the enzyme solution (109 μM in creatine kinase) contained non-radioactive ADP up to 2 mM and it was mixed in the quenched-flow apparatus with a solution of 20 mM-creatine phosphate and 2 mM radioactive ADP. The degree of saturation is estimated at 98%. (Although these high degrees of saturation were calculated, the activity is markedly higher in the conditions of the test solution.)

As shown in Fig. 2, again a straight line is produced (●) with a slope corresponding to $64\,s^{-1}$ (correlation coefficient = 0.97, standard deviation = 10 μM). There is no pronounced lag phase apparent in the formation of radioactive products, indicating that the intially bound ADP was completely exchanged with the radioactive nucleotide before the substrates are turned over, as expected for a rapid-equilibrium mechanism.

Although, in all the graphs, the correlation lines go through the origin, pointing to the identification of product formation as the rate-limiting step in both directions, the value of the standard deviation (10% of the active sites or 16% of the overall enzyme concentration, when the limited activity is taken into account) does not allow the exclusion of a small transient phase (in the case of a positive deviation) or a small lag phase (negative deviation). The value of the possible transient phase, however, allows one to say that, if product formation and the rate-limiting step are not identical, they are at least within the same order of magnitude. For a lag phase the same can be said for the rate-limiting step preceding the formation of the products. Hammes & Hurst (1969) found only isomerizations that were much faster than the turnover of the enzyme, which again points to the identification of the product formation as the rate-limiting step. It must be considered, however, that relaxation techniques based on the use of pH indicators only detect those steps that are accompanied by an exchange of protons.

It is clear that these conclusions are only valid at and around pH 8. In a more acid medium the reaction rate in the direction of ATP synthesis increases very much. It is not impossible that under these conditions the conformational change becomes rate-limiting. The reaction under these conditions is, however, too fast to be studied by quenched-flow techniques.

The behaviour of creatine kinase is in contrast with an increasing number of enzymes which were found to have a rate-limiting conformational change in their mechanism (Gutfreund, 1975). Such a conformational change is an excellent site for indirect control of activity and for the linkage of different enzyme systems. The fundamental role of creatine kinase seems to be to phosphorylate ADP rapidly so as to buffer the ATP concentration during its consumption in muscle contraction.

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References

Bagshaw, C. R. & Trentham, D. R. (1973) *Biochem. J.* **133**, 323–328

Glynn, I. M. & Chappell, J. B. (1964) *Biochem. J.* **90**, 147–149

Gutfreund, H. (1969) *Methods Enzymol.* **16**, 229–249

Gutfreund, H. (1972) *Enzymes: Physical Principles*, Wiley-Interscience, London

Gutfreund, H. (1975) *Prog. Biophys. Mol. Biol.* **29**, in the press
Hammes, G. G. & Hurst, J. K. (1969) *Biochemistry* **8**, 1083–1094
Holbrook, J. J. & Gutfreund, H. (1973) *FEBS Lett.* **31**, 157–169
Kuby, S. A., Noda, L. & Lardy, H. A. (1954) *J. Biol. Chem.* **209**, 191–201
Morrison, J. F. & Cleland, W. W. (1966) *J. Biol. Chem.* **241**, 673–683
Morrison, J. F. & James, E. (1965) *Biochem. J.* **97**, 37–52
Watts, D. C. (1973) *Enzymes*, 3rd edn., **8**, 384–456