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The Number of Catalytic Sites in Creatine Phosphokinase as Determined by a Study of its Reactive Sulphydryl Groups

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Rosenberg & Ennor (1955) demonstrated that creatine phosphokinase was readily inactivated by a variety of 'sulphydryl reagents', suggesting that free SH groups on the enzyme were essential for catalytic activity. Benesch, Lardy & Benesch (1955) showed that the enzyme contained six SH groups of which two are freely reactive. The presence of only two freely reactive SH groups was confirmed by Mahowald & Kuby (1960), who isolated the products, after acid hydrolysis, of the reaction with fluorodinitrobenzene and by Watts, Rabin & Crook (1961), who showed that alkylation of two reactive thiols by iodoacetate exactly parallels loss of enzyme activity. Since both these groups of workers reported that reaction of two SH groups was accompanied by complete loss of enzyme activity, it seemed reasonable to infer that creatine phosphokinase contained a maximum of two catalytic sites per molecule provided that the activity loss is not due to changes in the secondary and tertiary structure of the protein. This study of the kinetics of the reaction between creatine phosphokinase and iodoacetate was carried out to determine the number of catalytic sites per molecule of enzyme and the number of SH groups associated with each site. We have found that the properties of different crystalline preparations varied. Evidence is presented that a particular preparation of creatine phosphokinase may contain either one or two catalytic sites per molecule.

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MATERIALS AND METHODS

Abbreviation. CPK, Creatine phosphokinase.

Crystalline creatine phosphokinase. Two samples were prepared from rabbit muscle by the method B of Kuby, Noda & Lardy (1954) and the purity was checked by starch-gel electrophoresis at pH 8.9 by using the discontinuous system of Poulik (1957). Only one component and the trace of a second, which increased on storage, could be detected. A molecular weight of 81000 (Noda, Kuby & Lardy, 1954) was used to compute enzyme concentrations.

Iodoacetic acid. This was recrystallized four times from light petroleum, b.p. 60–80°, to give a white product, m.p. 78°, containing no titratable iodine.

Crystalline ATP (disodium salt). This was obtained from Sigma Chemical Co. and used without further purification.

Creatine. This was recrystallized from hot water.

p-Nitrophenylacetate. This was recrystallized first from hot 50% ethanol and then from sodium-dried ether. The final product, m.p. 80.5°, was dried over phosphorus pentoxide *in vacuo*.

Buffers. Borate buffer, 1.0 M, was prepared by adjusting 0.2 M-sodium hydroxide to pH 9.0 with boric acid and diluting the buffer to twice the original volume. The pH was checked at the final dilution. A pH 9.0 buffer from recrystallized glycylglycine was prepared by a similar method.

Ionic strength. Where necessary, this was adjusted by the addition of α -potassium nitrate.

Assay procedures. The reaction of iodoacetate with CPK was followed by measuring the production of iodide ions with the silver-silver iodide electrode. Kinase activity was determined by measuring the phosphate transferred from ATP to creatine in a standard reaction mixture in 4 min. at 37°. The details of these assays were exactly as set out by Watts, Rabin & Crook (1961).

RESULTS

Curve *AB*, Fig. 1, shows the progress curve of the reaction of iodoacetate with CPK in terms of moles of iodide liberated per mole of enzyme reacted. There is a rapid initial production of iodide which is completed in 40 min. leaving a slow secondary reaction. Correction for the secondary reaction by extrapolation to zero time, as shown by the dashed line (Fig. 1 *B₀B*), gives a value of *B₀* equal to 1.9 moles of iodide/mole of enzyme. This is presumably the total iodide produced in the primary reaction and represents 95% of two thiols reacting.

The effect of the substrate equilibrium mixture on this reaction is shown by curve *CD*, Fig. 1. The progress curve is still biphasic, but the primary reaction is greatly reduced. Extrapolation of this curve after 40 min. to zero time (Fig. 1 *D₀D*) now gives a value (*D₀*) of 1.03 moles of iodide/mole of enzyme. Control experiments showed that none of the components of the substrate equilibrium mixture reacted with iodoacetate at a detectable rate under the conditions used. Substitution of glycylglycine buffer (pH 9.0) for the borate buffer was without effect on these reactions.

The protective effect of the substrate equilibrium mixture against inhibition of kinase activity by iodo-

acetate is shown in Fig. 2. Conditions are the same as for Fig. 1 but for the omission of Tween 80, used to prevent the enzyme coating the iodide electrode, since it interferes with the phosphate assay.

Fig. 2. Reaction of creatine phosphokinase (sample 1) with iodoacetate at 25° as measured by loss of kinase activity in the presence and in the absence of the substrate equilibrium mixture. Conditions were as in Fig. 1 but without Tween 80. After the reaction was started with iodoacetate, samples (0.05 ml.) were transferred to 1.25 ml. of ice-cold 0.01 M-cysteine, pH 9.0, at the times shown. This rapidly removed unchanged iodoacetate so that the enzyme activity remaining could be determined by a standard assay. ●, Without equilibrium mixture; ○, plus equilibrium mixture.

Fig. 1. Reaction of creatine phosphokinase (sample 1) with iodoacetate at 25°, in the absence and in the presence of the equilibrium mixture of substrates, as measured by the production of iodide ions. ○, The reaction mixture contained: CPK, 3.3 μM; KI, 1 μM; Tween 80, 0.02% (v/v); borate buffer, pH 9.0, *I* 0.12; total volume, 5 ml. ●, The reaction mixture as above plus equilibrium mixture of substrates obtained by allowing the reaction in a mixture containing ATP and MgSO₄, 1 mM, and creatine, 10 mM, to proceed to equilibrium (15 min.) before adding the iodoacetate. For both experiments the reaction was started by the addition of 0.05 ml. of iodoacetate (final concn., 0.5 mM).

Fig. 3. First-order log plots from the data shown in Figs. 1 and 2. For experiments in which the iodide production was measured, *a* is the concentration of iodoacetate-reacting sites determined by extrapolation of the secondary region of the progress curve to zero time, and *x* is the molar concentration of iodide produced at any given time corrected as shown. Ordinates, $\log(a-x)$: ○, Curve *CD-B₀B* (Fig. 1), $a = B_0[\text{CPK}]$; ●, curve *AB-B₀B* (Fig. 1), $a = B_0[\text{CPK}]$; +, curve *CD-D₀D* (Fig. 1), $a = D_0[\text{CPK}]$. For activity-loss experiments, *a* is the initial molar concentration of enzyme, and *x* is the molar concentration of inactive enzyme after a given time, determined from the percentage decrease in activity (zero inhibition = 100% activity). Ordinates, $\log(a-x)-1$: ▲, Curve *A* (Fig. 2); △, curve *B* (Fig. 2).

Table 1. Apparent second-order velocity constants for the reaction of iodoacetate with the sulphhydryl groups of creatine phosphokinase (sample 1) in the presence and in the absence of the substrate-equilibrium mixture

Constant	k (l.mole ⁻¹ min. ⁻¹)
Overall constant (Fig. 1 $AB-B_0B$)*	360
In the presence of the equilibrium mixture	
(1) Rapidly reacting thiol (Fig. 1 $CD-D_0D$)	170
(2) Slowly reacting thiol (Fig. 1 D_0D-B_0B)	20
From loss of enzyme activity (Fig. 2A)	130
From loss of enzyme activity in the presence of the equilibrium mixture (Fig. 2B)	40

* Calculated on a molar enzyme basis. All other constants were calculated on a molar reactive-thiol basis.

Fig. 4. Inhibition of creatine phosphokinase (sample 1) by *p*-nitrophenylacetate at 25°. The reaction was started by the addition of *p*-nitrophenylacetate (final concn. 2 mM) and samples (0.1 ml.) were transferred to ice-cold, 0.01 M-cysteine, pH 9.0, which effectively stops the reaction. The enzyme activity remaining was then determined in a standard assay mixture (100% inhibition = zero kinase activity). Other constituents were: CPK, 2.4 μ M; borate buffer, pH 9.0, I 0.1.

Fig. 3a shows that the primary reaction of CPK with iodoacetate (Fig. 1 $AB-B_0B$) gives a linear first-order plot provided that a (the initial concentration of reactive enzyme) is set equal to B_0 (i.e. approximately twice the enzyme concentration). No corrections are necessary for the change in iodoacetate concentration during the course of the reaction because this is present in vast excess compared with the enzyme. The primary reaction in the presence of the substrate equilibrium mixture (Fig. 1 $CD-D_0D$) also gives a linear log plot (Fig. 3b) provided a is set equal to D_0 (i.e. the enzyme concentration). Fig. 3 (*d* and *e*) also shows that loss of enzyme activity is a simple first-order process under both sets of conditions.

The apparent second-order velocity constants of the two SH groups in the presence and in the

Fig. 5. Reaction of iodoacetate with creatine phosphokinase pretreated with *p*-nitrophenylacetate at 25°. ●, CPK, 8.95 μ M, pretreated with *p*-nitrophenylacetate, 6.7 mM, for 30 min. in borate buffer, pH 9.0. ○, Control, water used instead of *p*-nitrophenylacetate. In the subsequent reaction with iodoacetate the final concentrations of reactants were; CPK, 6.7 μ M; Tween 80, 0.02% (v/v); borate buffer, pH 9.0, I 0.12; total volume, 5 ml.

absence of the equilibrium mixture have been determined from the above data and are given in Table 1.

CPK activity is readily inhibited by *p*-nitrophenylacetate (Fig. 4). This inactivation is prevented and halted, but not reversed, by the addition of excess of cysteine. Fig. 5 shows that the initial production of iodide is much less rapid than the control and falls off after about 50 min. to a linear secondary reaction. Correction for the secondary reaction by extrapolation to zero time gives D_0 equal to 1 mole of iodide/mole of enzyme. Control experiments showed that neither *p*-nitrophenylacetate nor its hydrolysis products reacted with iodoacetate at the concentrations used. Table 2 lists the apparent second-order velocity constants of the SH groups under these conditions. The progress curve for the primary reaction after treatment with *p*-nitrophenylacetate was found to

Table 2. Apparent second-order velocity constants for the reaction of iodoacetate with the sulphhydryl groups of creatine phosphokinase (sample 1) pretreated with *p*-nitrophenylacetate

Constant	k (l.mole ⁻¹ min. ⁻¹)
Overall constant (Fig. 5 $AB-B_0B$)*	320
Rapid primary reaction after <i>p</i> -nitrophenylacetate treatment (Fig. 5 $CD-D_0D$)	150
Slow secondary reaction after <i>p</i> -nitrophenylacetate treatment (Fig. 5 D_0D-B_0B)	10

* See footnote to Table 1.

Fig. 6. Reaction of creatine phosphokinase with iodoacetate at 25°. Repeat of the experiments shown in Figs. 1 and 5 with sample 2 of CPK. ●, Control experiment; ○, plus substrate equilibrium mixture; △, CPK, 6-15 μ M, pretreated with *p*-nitrophenylacetate, 6-7 mM. The final concentrations for the reaction between CPK and iodoacetate were: CPK, 4-6 μ M; iodoacetate, 0-5 mM. Other conditions were as shown in Fig. 1 for the experiment with the substrate equilibrium mixture and Fig. 5 for the effect of *p*-nitrophenylacetate.

Fig. 7. Effect of the substrate equilibrium mixture on the reaction of creatine phosphokinase (sample 2) with iodoacetate. First-order log plots from the data shown in Fig. 6. a is the initial concentration of iodoacetate-reacting sites on the enzyme and is equal to twice the molar concentration of enzyme. ○, Curve $C-B_0B$ (Fig. 6); ●, curve $AB-B_0B$ (Fig. 6).

Fig. 8. Effect of *p*-nitrophenylacetate on the reaction of creatine phosphokinase (sample 2) with iodoacetate. First-order log plots from the data shown in Fig. 6. a is the initial concentration of the enzyme in terms of the iodide produced in the reaction with iodoacetate. ○, Curve $DE-B_0B$ (Fig. 6), $a = 2[\text{CPK}]$; ●, curve $DE-E_0E$ (Fig. 6), $a = 0.76[\text{CPK}]$.

give a first-order log plot if a was put equal to the initial concentration of enzyme.

All the experiments so far described were found to be quite reproducible for that particular preparation of CPK (sample 1). However, when the experiments were repeated with a new enzyme preparation (sample 2) different results were obtained. Fig. 6 shows the effect of the substrate equilibrium mixture on the reaction with iodoacetate. The control reaction proceeded as before (Fig. 6A,B) yielding 2 moles of iodide/mole of CPK, with an apparent second-order velocity constant of 180 l. mole⁻¹ min.⁻¹ for the two SH groups. In the presence of the equilibrium mixture the reaction rate was depressed (Fig. 6C) but, unlike sample 1, did not become markedly biphasic. Fig. 7 shows first-order log plots of the curves A and C after correction for the secondary reaction, B_0B of Fig. 6. It can be seen that, unlike sample 1 (Fig. 3c), the plot in the presence of the equilibrium mixture is linear over the whole of the measured range and both sites reacting with iodoacetate are modified to the same extent.

When CPK (sample 2) was treated with *p*-nitrophenylacetate, enzyme activity was lost as before and the progress curve of the enzymically inactive protein with iodoacetate was biphasic

(Fig. 6DE). However, extrapolation of the secondary reaction to zero time gave a value (E_0) of 0.76. This would correspond to a primary reaction involving 38 % of the two SH groups. A first-order log of curve DE minus the secondary reaction of the control (curve B_0B , Fig. 6) was markedly curved over the first 40 min. of the reaction (Fig. 8a) but that for the primary reaction alone (curve $DE-E_0E$, Fig. 6) was linear (Fig. 8b).

DISCUSSION

The two thiols in CPK possess approximately equal reactivity. This is clear from the work of Rabin & Watts (1960) in which it was found that the total activity of the two thiols remained constant over the pH range 6–10, and in the present work in which a first-order log plot of the primary reaction with iodoacetate is linear (Fig. 3a). If the thiols had possessed markedly different reactivities towards iodoacetate then the progress curve would have been biphasic and the first-order log plot derived from it non-linear. This is clear from the experiments with sample 1 in which the substrate equilibrium mixture alters the relative reactivities of the two thiols (Figs. 1CD and 3c). Linearity of the first-order log plot is a necessary condition for the identity of the species reacting with iodoacetate; it would be unwise to regard it as a sufficient condition.

Watts, Rabin & Crook (1961) showed that, in the reaction of iodoacetate with CPK, activity loss exactly parallels the alkylation of the two reactive thiols. This eliminates the possibility of a single catalytic site containing two SH groups both essential for catalytic activity, since the rate of loss of enzyme activity, on a percentage activity basis, would have been initially twice that of the rate of alkylation of the two thiols. The remaining possibilities that must be considered are that the enzyme has two catalytic sites, each containing one reactive thiol, or it possesses a single catalytic site containing one reactive thiol, the other being elsewhere on the protein and not associated with enzyme activity. The second possibility implies that the chemical environments of the two thiols are different since only one of them is at the catalytic site. By selective treatment of the enzyme it should be possible to modify the activity of one of them so as to produce a biphasic inhibitor progress curve. It was possible to do this with sample 1 of CPK (Figs. 1 and 5). The catalytic-site thiol was selectively protected by an equilibrium mixture of substrates and selectively inhibited by *p*-nitrophenylacetate. A detailed analysis of the data upholds this viewpoint.

The corrected curve for the rapid production of iodide in the absence of the equilibrium mixture

(Fig. 1AB– B_0B) yields an overall apparent second-order velocity constant of 360 l.mole⁻¹ min.⁻¹ for both thiols. Since both thiols possess approximately equal reactivity the velocity constant in terms of sulphhydryl-group concentration (rather than enzyme concentration) is 180.

In the presence of the equilibrium mixture the initial reaction with iodoacetate is much slower (Fig. 1C). The progress curve, corrected for the same secondary reaction as in the control, is biphasic, and the first-order log plot is markedly curved (Fig. 3c). Extrapolation of the second part of the progress curve to zero time (Fig. 1 D_0D) yields 1 mole of iodide/mole of enzyme, suggesting that this treatment distinguishes the two thiols. Correction of curve CD (Fig. 1) by subtraction of the extrapolated portion (Fig. 1 D_0D) gives the progress curve for the thiol unaffected by the equilibrium mixture. This should follow first-order kinetics and have a velocity constant equal to that calculated above, provided that its reactivity is completely unaffected by the equilibrium mixture. Fig. 3b shows that the log plot of the corrected curve is linear and an apparent second-order velocity constant of 170 is obtained.

Fig. 2 shows that the equilibrium mixture protects the thiol at the catalytic site from iodoacetate. Both enzyme inhibition curves give linear log plots (Fig. 3d and e) from which a value for the velocity constant of the catalytic-site thiol of 130 was obtained. The sum of the constants for the catalytic- and non-catalytic-site thiols (300 l. mole⁻¹ min.⁻¹) should equal the overall constant (360 l. mole⁻¹ min.⁻¹). In the light of the assumptions that have to be made and the low precision of the phosphate assay this agreement is satisfactory.

The curve for the secondary reaction in the presence of the equilibrium mixture, corrected for the secondary reaction of the control (i.e. Fig. 1 D_0D – B_0B), should approximate to the progress curve for the catalytic-site thiol in the presence of the equilibrium mixture. From this an apparent second-order velocity constant of 20 l. mole⁻¹ min.⁻¹ was obtained. Considering the unsatisfactory method of calculation, this is in satisfactory agreement with the constant obtained from loss of enzyme activity (Table 1). This clearly shows that protection of the enzyme against iodoacetate by the substrate equilibrium mixture can only be associated with blocking one thiol since the reactivity of the other is hardly affected (Table 1).

This finding of one catalytic site per molecule of CPK in which only one thiol is involved is further supported by the results from the reaction of iodoacetate with CPK which has been pretreated with *p*-nitrophenylacetate. The enzyme is rendered catalytically inactive (Fig. 4), yet 1 equiv. of iodide/mole of enzyme is rapidly produced by

reaction with iodoacetate (Fig. 5), showing that only one thiol has been rendered unreactive by *p*-nitrophenylacetate. The apparent second-order rate constant of the thiol remaining (Table 2) is of the same order as that for the singly reacting thiol in the presence of the substrate equilibrium mixture.

These experiments with sample 1 of CPK were found to be completely reproducible with that enzyme preparation. Sample 2 showed a different and contrasting behaviour. The substrate equilibrium mixture protects the enzyme against iodoacetate without causing the progress curve to become markedly biphasic, and a linear first-order log plot (Fig. 7*b*) of the corrected progress curve (Fig. 6*C-B₀B*) is obtained in contrast with that of the first sample (Fig. 3*c*). Thus for sample 2 the activities of both thiols have been depressed to the same extent by addition of the equilibrium mixture. This provides strong evidence for the existence of two catalytic sites per molecule in this sample of CPK. The reaction of sample 2 with *p*-nitrophenylacetate is obviously complex and the subsequent reaction with iodoacetate (Fig. 6*DE*) does not allow any definite conclusions to be drawn concerning the identity of the two thiols.

It is clear that samples of CPK containing either two or one catalytic site per molecule can be obtained. Not more than one SH group is associated with each catalytic site. It is possible that the enzyme with only one site may be formed by a partial unfolding of the protein. This could separate the thiol from the substrate-binding sites without impairing the individual properties of either. Such a modification of the protein could occur as a result of the rather harsh isolation procedure or be of genetic origin. So far, only a single preparation containing one catalytic site has been obtained. Two subsequent preparations, in addition to the two reported here, have both behaved in the manner associated with two catalytic sites. Attempts to modify CPK by heat and ethanol treatment so as to produce one catalytic site per molecule have been unsuccessful, but this may be because it is much more difficult to modify the enzyme after it has been highly purified.

If the variation in CPK is of genetic origin then it might be necessary to investigate many samples of the enzyme before finding another with only one catalytic site. Genetic effects of this kind are not unknown. For example, the glutamic dehydrogenase of a mutant of *Neurospora crassa* has differences from the wild-type enzyme which have been attributed to changes in the tertiary structure of the protein (Fincham, 1957).

The evidence of Kuby & Mahowald (1959) supports the idea of two substrate-binding sites, but would not distinguish a substrate-binding site

that had been separated from the catalytic site, the protein being otherwise unimpaired.

From the present experiments it is only possible to conclude that most samples of CPK contain two catalytic sites per molecule of enzyme but that preparations with only one catalytic site can be obtained. The time required to prepare pure samples of CPK and carry out the necessary experiments precludes a large-scale search for a genetic variant at the present time.

SUMMARY

1. Creatine phosphokinase reacts rapidly with iodoacetate to produce 2 equiv. of iodide as determined with the iodide electrode. Complete enzyme inactivation results and is associated with the alkylation of two SH groups.

2. The possibility of one catalytic site with two SH groups essential for activity is excluded.

3. With one sample of creatine phosphokinase the substrate equilibrium mixture protects one SH group but not the other. This indicates one catalytic site containing one essential SH group.

4. This view is supported by the reaction of creatine phosphokinase with *p*-nitrophenylacetate in which the catalytic site SH group is selectively inhibited as judged by the subsequent reaction with iodoacetate.

5. The SH groups of a second sample of creatine phosphokinase are equally affected by the substrate equilibrium mixture and by *p*-nitrophenylacetate. This suggests two catalytic sites with one SH group at each for this preparation.

6. The possibilities that the differences in the two preparations are of genetic origin or result from the isolation procedure are discussed.

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