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THE NATURE AND REACTIVITY OF THE 'ESSENTIAL' THIOL IN RABBIT MUSCLE CREATINE KINASE III (EC 2.7.3.2)

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Summary: Rabbit muscle creatine kinase III (EC 2.7.3.2) can be reacted with 2-chloromercuri-4-nitrophenol and this results in the incorporation of two moles of mercurial per mole of enzyme subunit in a biphasic reaction. The second-order rate constant for the slow reaction is 475 ± 42 M^{-1} s^{-1}. S-Carboxamidomethyl-creatine kinase reacts with a single mole of mercurial per mole of subunit. The rate constant, 466 ± 57 M^{-1} s^{-1}, is almost identical to that for the slow reaction of the native enzyme. The reaction between 3-carboxy-4-nitrophenylthio-creatine kinase and 2-chloromercuri-4-nitrophenol has a second-order rate constant of 449 ± 56 M^{-1} s^{-1}. The results may be explained if the mercurial reacts very rapidly with that cysteine residue which reacts independently with iodoacetamide or 5,5'-dithiobis(2-nitrobenzoic acid). However, 2-chloromercuri-4-nitrophenol also reacts more slowly with a second cysteine residue. Definition of the essentiality of thiol groups in enzymes by reaction with *labile ligands*, here represented by organomercurials, clearly must be approached with caution.

The interpretation of experiments involving the reaction of rabbit muscle creatine kinase III (EC 2.7.3.2) with thiol-specific reagents has remained a problem for many years. Many inhibitors have been used, some apparently leading to complete inhibition (1-11), others to at least partial retention of activity (12-15).

There has been considerable uncertainty regarding the location of the substituent after reaction. In some experiments, a thiol distinct from the 'essential' thiol is reported to have been modified (14-16). Little attention has been paid to the possibility of exchange and/or displacement of modifying groups before and during assays for residual activity.

Der Terrossian and Kassab (12) postulated the presence of two thiol groups in such close proximity that modification of the first by anything bulkier than cyanide sterically prevented modification of the second thiol. In this Communication, we report results which discount this proposition and explain the reactions of two (presumably juxtaposed) thiols at the active site of the enzyme with a variety of thiol-specific reagents.

Experimental Section

Rabbit muscle creatine kinase III from two sources was used. Enzyme purchased from Boehringer Mannheim had a maximum specific activity of 1,934 μkat/g. Later, enzyme was isolated according to Noda *et al.* (17). The recrystallisation procedure was not used. Fresh enzyme had a specific activity of 2,100 μkat/g, but this decreased on storage. Enzyme of specific activity 1,800 – 2,000 μkat/g was used for the experiments below.

Enzyme activities were measured using a Radiometer pH Stat assembly at pH 8.8 and 30°C. The reaction mixture contained 40 mM creatine, 4 mM ATP, 5 mM magnesium acetate, 0.1 mM dithiothreitol and 0.05 mM EDTA. In situations where dithiothreitol would cause unwanted reactivation of the enzyme, 0.1 mM EDTA was used instead.

Spectrophotometric titration of fresh enzyme with DTNB indicates that the maximum specific activity for rabbit muscle creatine kinase III is ~2,300 μkat/g, a result consistent with a number of previous reports (3, 18-20).

Stock solutions of enzyme with fully reduced cysteine residues were prepared by dialysis against oxygen-free 0.1 M Bicine-NaOH buffer, pH 8.01 (1 mM each in EDTA and dithiothreitol). This was followed by exhaustive dialysis under oxygen-free conditions against the same buffer without dithiothreitol. The concentration of reactive thiols was determined by titration with DTNB.

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TABLE I

Second-order Rate Constants for the Slow Reaction of Creatine Kinase III with CMNP at pH 8.01, 25°C

$[CMNP]_o/[Enzyme\ subunit]_o$	$[Enzyme\ Subunit]_o$ (μM)	k ($M^{-1} s^{-1}$)
2.03	8.74	529 ± 9
2.11	8.13	482 ± 1
2.30	7.89	525
2.37	8.12	480 ± 6
2.63	8.11	459 ± 6
3.51	8.69	472
3.76	7.94	413 ± 6
3.95	8.07	431

All experiments involving 2-chloromercuri-4-nitrophenol (CMNP)[1] were performed in oxygen-free 0.1 M Bicine-NaOH, pH 8.01 (1 mM in EDTA). The stock solution of CMNP (m.p. 239.5°C) was made up in 0.1 M NaOH at a concentration of 2 mM.

The kinetics of the reaction of CMNP with native enzyme at 25°C were monitored at 416.8 nm using a Cary 17 recording spectrophotometer.

Carboxamidomethylated enzyme was prepared by reaction of creatine kinase with iodoacetamide, after which excess reagent was removed by dialysis. Reaction of the modified enzyme with CMNP was followed at 416.5 nm.

Native enzyme was reacted with an excess of DTNB and then chromatographed on Sephadex G-25 to remove DTNB and released TNB. The modified enzyme was then reacted with varying aliquots (2-10 μl) of the stock CMNP solution. The spectral changes were observed at 416.8 nm and the kinetics of the reaction with CMNP were analysed.

A corresponding experiment was attempted in which enzyme was reacted with a stoichiometric amount of CMNP, and the kinetics of the reaction between this enzyme and an added excess of DTNB were examined.

The concentration of residual thiols in enzyme derivatives was determined by titration with DTNB in buffer containing 6 M GH^+Cl^-(21,22). Stoichiometry of incorporation of mercurial was determined by atomic absorption spectrometry using a Varian AA-6 instrument.

Results

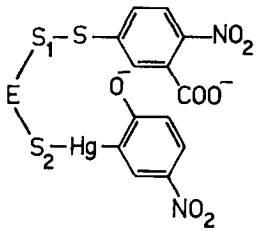
The reaction of native creatine kinase with one equivalent of CMNP per subunit is very rapid (23,24), and has $\Delta\epsilon_{416.8}$ = 17,400 ± 500 M^{-1} cm^{-1}. The reaction of a second equivalent is slower. The $\Delta\epsilon$ at 416.8 nm after completion of the reaction with two equivalents of mercurial is 37,300 ± 1,100 M^{-1} cm^{-1}. The second-order rate constants for the reaction of the enzyme with CMNP as CMNP concentration varies are shown in Table I. Analysis of the second-order reaction is complicated by a very slow apparently first-order absorbance change with a rate constant of approximately 4 x 10^{-4} s^{-1}. This may be due to a very slow reaction of the mercurial with other thiols, but in any event, appropriate correction of the experimental data yields the second-order rate constants in Table I.

Enzyme samples treated with one and two equivalents of CMNP per subunit were dialysed to remove free reagent and analysed by atomic absorption spectrometry. The results indicate 0.87 and 1.94 g-atom of mercury incorporated per mole of subunit. DTNB titrations of these modified enzyme samples in the presence of 6 M GH^+Cl^- yield results commensurate with the loss of one and two titratable thiols per subunit respectively.

[1] The following abbreviations have been used: Bicine, N,N-bis(2-hydroxyethyl)glycine; CMNP, 2-chloromercuri-4-nitrophenol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); E-CH_2CONH_2, a creatine kinase subunit modified by reaction with iodoacetamide; E-$(MNP)_1$, an enzyme subunit modified by reaction with one equivalent of CMNP; E-$(MNP)_2$, an enzyme subunit modified by reaction with two equivalents of CMNP; E-TNB, enzyme modified by reaction with DTNB; GH^+Cl^-, guanidinium chloride.

Only one equivalent of CMNP reacted with carboxamidomethylated enzyme. The reaction, monitored at 416.5 nm, was second-order, and $\Delta\epsilon_{416.5}$ was 19,900 ± 1,000 M^{-1} cm^{-1}. Atomic absorption spectrometry and DTNB titration in the presence of GH^+Cl^- confirmed the incorporation of one equivalent of mercurial per subunit. In experiments where the concentration of CMNP was one to five times the enzyme subunit concentration, the rate constant was 466 ± 57 M^{-1} s^{-1}.

When E-TNB was reacted with varying amounts of mercurial, the spectral change at 416.8 nm was second-order, with a rate constant of 449 ± 56 M^{-1} s^{-1} at pH 8.01 and 25°C. The results were consistent with the incorporation of one equivalent of mercurial per enzyme subunit. We suggest that the product of this reaction has the structure (I), where E represents the enzyme subunit, S_1 is the 'essential' thiol and S_2 the second thiol; $\Delta\epsilon_{416.8}$ for the reaction was 19,040 ± 500 M^{-1} cm^{-1}. It is necessary to remove free TNB from the E-TNB preparations because TNB reacts with CMNP.



(I)

When E-$(MNP)_1$ was reacted with excess DTNB, the reaction showed first-order kinetics independent of [DTNB], and was apparently consistent with the occurrence of two independent first-order reactions yielding the same product (25). It was expected that the product spectrum would be the same as that arising from structure (I). However, it became obvious that the free TNB released in this experiment was competing successfully with the enzyme thiols for the available mercurial. A study of this reaction in the absence of free TNB is not experimentally accessible, but since DTNB is reported to react only with $cysteine_1$, the fact that a reaction is occurring at all is evidence for the exchange of mercurial between $cysteine_1$ and $cysteine_2$.

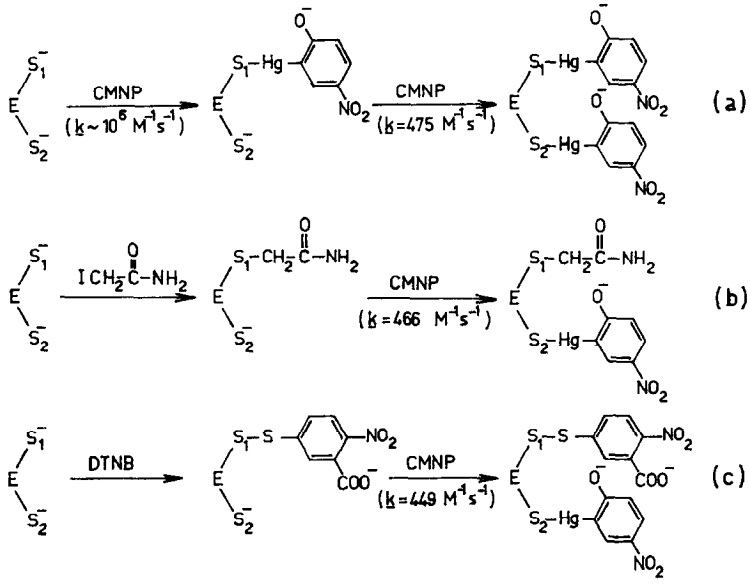
Discussion

The results obtained may be considered in terms of Scheme 1. We propose that the initial fast reaction occurring between creatine kinase and CMNP is with the 'essential' thiol [(a)]. Prior alkylation with iodoacetamide or reaction with DTNB prevents the fast reaction with this thiol [(b), (c)]. These rate constants indicate that CMNP is able to react directly with the second thiol. This thiol does not normally react with thiol-specific reagents (1-11).

The virtually identical values of the second-order rate constants in these three cases indicate that the reaction is occurring with the same thiol group. *This is not the 'essential' thiol because reaction still occurs after* $cysteine_1$ *has been blocked with DTNB or iodoacetamide to yield 100% inactive enzyme.*

These results are in direct contrast with the conclusions of Der Terrossian and Kassab (12) and also those of Laue and Quiocho (15) who assumed that the initial fast reaction of CMNP was with $cysteine_2$.

Khalifah and his co-workers (26,27) have recently reported the very facile exchange reactions between potential ligands of organomercurials. The rate constant for the reaction of glutathione and CMNP is reported to



Scheme 1

be altered dramatically when the labile ligand is varied. Rabenstein and Evans (28) also report the rapid exchange of methylmercuric ion among available free thiols.

The results of the experiment with E-$(MNP)_1$ and DTNB serve to highlight the caution necessary when attempting to establish the essentiality or nonessentiality of residues for enzyme catalysis. The possibility that exchange or displacement of substituting groups is the basis for the finite residual activity of modified creatine kinase species has not been considered carefully in the past. Thus the (non)essentiality of active site thiols in creatine kinase is still open to question.

Acknowledgements

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