Properties of a CH₃S-blocked Creatine Kinase with Altered Catalytic Activity

KINETIC CONSEQUENCES OF THE PRESENCE OF THE BLOCKING GROUP*

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Steady state kinetic parameters for rabbit muscle creatine kinase (EC 2.7.3.2) and this enzyme stoichiometrically blocked at the iodoacetamide-sensitive cysteiny1 residue with a CH₃S- group have been measured at 30 ± 0.1°, pH 9.00, using Mg(II) as the required metal ion cofactor. The double reciprocal plots for the CH₃S-blocked enzyme with MgATP as the variable substrate are biphasic, each curve showing a break at ~1.9 mM MgATP, and suggest the possibility of negative cooperativity in metal-nucleotide binding. Furthermore, extrapolated lines at high MgATP concentrations intersect on the abscissa, indicating loss of synergism in binding of substrates. In contrast, observed Michaelis constants for creatine are, within experimental error, the same for both native and blocked enzymes. The maximal velocity of the CH₃S-blocked enzyme is found to be 28.1% of the value of the native enzyme.

Product inhibition patterns for both native and blocked enzyme are also compared. Again, these patterns indicate that the CH₃S-blocking group alters the nucleotide binding site more than the guanidino substrate binding site. Calculations using the methods of Chou and Fasman ((1970) Biochemistry 13, 211-222) lead to the prediction that the active cysteinyl residue occurs at the beginning of a β turn which separates two portions of β sheet structure of the enzyme, and so may be in a position to mediate conformational changes in the protein.

Rabbit muscle creatine kinase has only one highly reactive cysteinyl residue per subunit (3). This sulfhydryl group has been blocked using numerous reagents, including iodoacetate (3, 4), iodoacetamide (5), 2,4-dinitrofluorobenzene (3), disodium tetrathionate (6), 5,5'-dithiobis (2-nitrobenzoic acid) (7, 8), and the spin label N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl) iodoacetamide (9, 10). In all of these cases, the authors reported that modification of this sulfhydryl group leads to essentially complete inactivation of the enzyme. In contrast, blocking this sulfhydryl group with the relatively small CH₃S- group leads to enzyme with substantial (~20%) residual enzymatic activity (8, 11). This residual activity provides a unique opportunity to examine the kinetic consequences of chemical modification of this important amino acid residue. In the present study a direct comparison is made between various steady state kinetic parameters of native and CH₃S-blocked enzymes. In the preceding paper (1) proton relaxation rate enhancements and EPR spectra of native, CH₃S-blocked, and iodoacetamide-inactivated creatine kinase are compared for a variety of ternary and quaternary enzyme substrate complexes using the paramagnetic Mn(II) ion as a spectroscopic probe.

MATERIALS AND METHODS

ATP, ADP, creatine, phosphocreatine, and bovine serum albumin were obtained from Sigma. Rabbit muscle creatine kinase (EC 2.7.3.2) was obtained from Calbiochem. Sephadex G-25 medium was purchased from Pharmacia. Methyl methanethiosulfonate was prepared as previously described (8). All other reagents were obtained from commercial sources.

Creatine kinase assays were carried out on a Radiometer TTT2 pH-stat using a modification of the procedure of Mahowald et al. (3). The water-jacketed reaction vessel was maintained at 30 ± 0.1° by circulating water from a constant temperature bath. A stream of N₂ gas, presaturated with water, was admitted just above the surface of the assay mixture to prevent absorption of atmospheric CO₂. The pH was maintained at pH 9.00 by automatic addition of 2.43 mM NaOH. The NaOH concentration was periodically checked by titration against potassium acid phthalate. The total Mg(II) concentration in the assay solution was adjusted to maintain the free Mg(II) at 1.0 mM, and the total ionic strength was adjusted to 50 mM by addition of varying amounts of sodium acetate. The concentration of creatine kinase was determined spectrophotometrically at 280 nm using an
extinction coefficient of 8.96 for a 1% solution and a molecular weight of 82,000 (12). Methanethiolation of creatine kinase was carried out as previously described (8, 11) at an [inhibitor] to [enzyme] (dimer) ratio of 5. After removal of excessive methyl methanethiol sulfonate by gel filtration on Sephadex G-25, the enzyme solution was stored at 4°C for 20 to 40 hr before the kinetic assays were conducted. The enzyme remained iodoacetamide-insensitive (6) even when stored in this manner for several days.

Previous kinetic and isotopic exchange studies by Morrison et al. (10-15) have established a kinetic scheme for rabbit muscle creatine kinase as shown in Scheme 1.

The data shown in Figs. 1 and 2 were analyzed by obtaining least squares fits to the equation for the rapid equilibrium, random, bimolecular-bimolecular rate equation (16) shown in

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v = \frac{V[MgATP][Cr]}{K_a[MgATP][Cr] + K_a[Cr][MgATP] + [MgATP][Cr] + K_a[MgATP]K_a[Cr]}\]  

Equation 1 where \(v\) is the observed velocity, \(V\) is the maximal velocity, \(Cr\) is creatine, and \(K_a\) and \(K_p\) refer to the kinetic parameters shown in Scheme 1. Data analysis was made using the computer program SEQUEN generously supplied by Dr. W. W. Cleland and modified by Dr. E. C. Gordon for use with the IBM 360 computer. It should also be noted that since \(K_p\) (MgATP) \(K_a\) (Cr), \(K_p\) (Cr) \(K_a\) (MgATP) determination of any three kinetic constants gives the fourth.

RESULTS AND DISCUSSION

Double reciprocal plots for ATP and creatine for native and CH,S-blocked creatine kinase are shown in Figs. 1 and 2, respectively. The double reciprocal plots for native creatine kinase (Fig. 1, a and b) are linear, and the extrapolated lines intersect above the abscissa, indicating synergism in binding of MgATP and creatine as first shown by Morrison and James (13). The kinetic parameters obtained at pH 9.00 for the native creatine kinase compare favorably with those determined by Morrison and James (15) obtained at pH 8.0.

The double reciprocal plots for CH,S-blocked creatine kinase with ATP as the variable substrate are biphasic, each curve showing a break at \(~1.9\) mM MgATP (Fig. 2b). In accord with these plots the double reciprocal plot for the CH,S-blocked enzyme with creatine as the variable substrate (Fig. 2a) shows two families of lines, with lines for \(~1.9\) mM MgATP intersecting above the abscissa and lines for \(~1.9\) mM MgATP intersecting on the abscissa.

Two possible explanations for the breaks in double reciprocal plots of Fig. 2b are (a) negative cooperativity in metal-nucleotide binding; (b) a heterogeneous population of enzyme forms with different properties. The following arguments support a chemically homogeneous modification: (a) when enzyme was modified with excess [methyl-14C]methanethiol sulfonate, 1:1 stoichiometry was observed; i.e. 1 eq of CH,S was incorporated/subunit (11); (b) the fully blocked enzyme exhibits a constant specific activity over a period of several days at 4°C; (c) the residual activity remained iodoacetamide-insensitive throughout this period, whereas iodoacetamide treatment normally completely inactivates the enzyme (8, 11); (d) EPR spectra for the complex of CH,S-blocked creatine kinase with MnADP, creatine, and nitrate showed that no detectable amount (i.e. less than 5%) of native enzyme-like complex was present (1); (e) EPR spectra for the complex of modified enzyme with MnADP, creatine, and thiocyanate indicated a homogeneous response of the enzyme to thiocyanate binding (1).

Another potential source of microheterogeneity, which cannot be ruled out, arises from hindered rotation about the newly formed -S-S- bond. Typical rotational barriers for -S-S- bonds in simple acyclic disulfides are only -7 kcal/mol (17). The enzyme may, however, introduce additional steric hindrance to this rotational process.

The possibility also remains that the CH,S group modifies a group (or groups) which, when blocked, confer protection of the normally iodoacetamide-sensitive sulfhydryl group. It is unlikely that such a hypothetical group is other than a sulfhydryl, since methyl methanethiol sulfonate exhibits a high degree of specificity for sulfhydryl groups of proteins in the presence of other side chain residues (9). Rapid removal of the CH,S group on creatine kinase with 2-mercaptopropanol, which fully restores the properties of the native enzyme, also supports the contention that a sulfhydryl group is blocked (8, 11). Furthermore, stoichiometric blocking of at least one of the other three sulfhydryl groups per subunit using organomercurials leads to no observable loss of catalytic activity, and leaves the enzyme completely sensitive to inactivation by iodoacetamide (11, 18, 19).

To examine the hypothesis that the breaks in the double reciprocal plots of Fig. 2b represent negative cooperativity, the appropriate kinetic data have been analyzed using the Hill equation (20). Hill coefficients were determined at various concentrations of creatine (data of Fig. 2) and extrapolated to infinite creatine concentration. A Hill coefficient of 0.59 at infinite creatine concentration was calculated. This value is consistent with negative cooperativity in this two subunit enzyme.
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Fig. 1. Double reciprocal plots for native rabbit muscle creatine kinase at pH 9.00, 30°. Assays were carried out under a nitrogen atmosphere using $2.20 \times 10^{-3}$ mg/ml of creatine kinase in the presence of 0.1% bovine serum albumin. The free Mg(II) concentration was kept constant at 1.0 mM and the ionic strength was adjusted to 50 mM using sodium acetate.

Negative cooperativity in metal nucleotide binding to rabbit muscle creatine kinase as reported previously (21) was expressed only in the presence of both creatine and nitrate. The cooperative behaviors of several other enzymes are influenced by effector binding (22), and the CH$_3$S-blocking group may be producing a similar phenomenon. For example, rabbit muscle pyruvate kinase exhibits cooperative behavior only in the presence of phenylalanine.

Fig. 2. Double reciprocal plots for CH$_3$S-blocked creatine kinase at pH 9.00, 30°. Assays were carried out exactly as described in legend for Fig. 1 except that the concentration of CH$_3$S-blocked enzyme was $1.02 \times 10^{-3}$ mg/ml.
observed with the native enzyme. Lines in Fig. 2b extrapolated from higher MgATP concentrations (>1.9 mM) intersect close to or on the abscissa, suggesting a substantial decrease in the synergism in MgATP and creatine binding. Statistical analysis of the kinetic data (cf. Table I) confirms this loss of synergism. Comparison of maximal velocities (expressed as V_max or turnover number) for the native and CH₃S-blocked enzymes shows that the true maximal velocity of the modified enzyme is 28.1% of the value of the native enzyme, although the curvature of the double reciprocal plot would lead to an estimate of only 19.9% of the native enzyme value if only low MgATP (<1.9 mM) concentrations were examined.⁴

Fig. 3 shows intercept plots for MgATP for both native and CH₃S-blocked creatine kinase. Also shown are extrapolated lines from data points obtained at high (>1.2 mM) and low (<1.2 mM) MgATP for the CH₃S-blocked creatine kinase. The figure shows that, within experimental error, the observed value for kcat (creatine) is unaltered by the presence of the CH₃S-blocking group.

Product Inhibition - Product inhibition patterns can distinguish among mechanistic schemes having rate equations of identical form. Fig. 4, a and b, shows selected product-inhibition patterns for both native and CH₃S-blocked creatine kinase. Secondary plots (not shown) are all linear and were used to evaluate inhibition constants. MgADP is competitive with respect to MgATP and phosphocreatine is competitive with respect to creatine. These observations are consistent with a rapid equilibrium, random, sequential mechanistic scheme for both native and blocked enzyme. The results are summarized in Table II. For both enzymes the rate-determining step is the interconversion of the ternary E·MgATP·creatine and E ∙ MgADP · phosphocreatine complexes. The kinetic constants reflect binding affinities of metal nucleotide and guanidine substrates and kcat is the rate constant for interconversion of the ternary complexes.

Phosphocreatine shows only a slight increase in K_m from the value found for the native enzyme. This is similar to the lack of any significant difference of K_m of creatine between the native enzyme and the CH₃S-blocked creatine kinase at either low or high ATP concentration. On the other hand, the nucleotide binding site is apparently somewhat altered since K_m (MgADP) for the CH₃S-blocked enzyme is 3-fold greater than for the native enzyme.

Synergism in substrate binding is presumably associated with substrate-induced conformational changes within the enzyme's tertiary structure. In the presence of the CH₃S-blocking group this synergism is lost at high MgATP concentrations; K_m (creatine) decreases 2-fold to become equal to K_m (creatine) and approaches K_m (creatine) for the native enzyme. Thus it appears that in the presence of the CH₃S-blocking group the conformation resulting from the binding of MgATP at one subunit may be transmitted to the adjacent protomer. This conformational change leads to weakening of the binding of MgATP since both K_m (MgATP) and K_m (MgATP) are increased at high MgATP concentrations, relative to the native enzyme. Furthermore the affinity for MgADP is diminished slightly by modification, as was found for MnADP and free ADP by proton relaxation rate titrations (1). Thus the majority of the effects of the CH₃S- label are reflected in the properties of the metal-nucleotide binding site.

Structural Predictions - Some insight into a possible structural role for the iodoacetamide-sensitive sulfhydryl group in native and blocked enzymes emerges using Chou-Fasman calculations (26, 27). These calculations allow prediction of secondary structural features in globular proteins using empirically determined probabilities of occurrence of amino acid residues in regions of secondary structure; e.g. α helix, β sheet, and β turns. This method predicts the occurrence of 88% of the helical regions and 95% of the β sheet regions in the 19 proteins evaluated by Chou and Fasman (27).

⁴ In preliminary investigations of Dr. D. J. Smith (23), methyl methanethiosulfonate treatment of a creatine kinase isoenzyme isolated from the cytoplasm of beef heart (24) led to a CH₃S-blocked enzyme whose V_max was 23 ± 2% that of the native enzyme. In addition, K_m values for creatine and MgATP were only slightly altered. Intense clinical interest in the creatine kinase isoenzyme from heart tissue in the monitoring of myocardial infarctions (25) prompted our investigations of its comparative behavior to that of the rabbit muscle isoenzyme.

TABLE I

| Kinetic parameters determined for native and CH₃S-blocked rabbit muscle creatine kinase |
|---------------------------------|---------------------|---------------------|---------------------|
|                                | Native creatine kinase | CH₃S-blocked creatine kinase |
| K_m (creatine)                 | 8.61 ± 0.57 mM       | 9.09 ± 1.35 mM       |
| K_m (ATP)                      | 0.25 ± 0.04 mM       | 0.31 ± 0.06 mM       |
| V_max (creatine)               | 24.4 ± 5.5 mM        | 16.0 ± 4.83 mM       |
| V_max (ATP)                    | 0.71 ± 0.10          | 0.65 ± 0.15 mM       |
| Efficent activity              | 136.5 ± 2.9 μmol/min/mg | 27.1 ± 2.05 μmol/min/mg |
| Residual activity              | (19.9%)              | (28.1%)              |
| high ATP, >1.9 mM              |                      |                     |
| K_m (creatine)                 | 10.16 ± 1.48 mM      |                     |
| K_m (ATP)                      | 1.27 ± 0.22 mM       |                     |
| K_c (creatine)                 | 9.32 ± 3.4 mM        |                     |
| K_c (ATP)                      | 1.16 ± 0.40 mM       |                     |
| V_max                           | 38.4 ± 1.34 μmol/min/mg | 28.1%            |

* Kinetic parameters were obtained from data on Figs. 1 and 2 by computer analysis as described under "Results and Discussion."
Product inhibition of native and CH₃S-blocked creatine kinase

| Concentration of fixed substrate | Type       | Kᵣ (MgADP) | Kᵢ (phosphocreatine) |
|----------------------------------|------------|------------|-----------------------|
| Native creatine kinase           | 0.48 mM    | 32 mM creatine | Competitive           |
| CH₃S-blocked creatine kinase     | 1.04 mM    | 32 mM creatine | Competitive           |

The amino acid sequence surrounding the iodoacetate-sensitive cysteinyl residue of creatine kinase is: Ala-Gly-Pro-His-Phe-Met-(Asp,His,GlU)-(Gly,Leu)-Tyr-Val-Leu-Thr-Cys-Pro-Leu-Gly-Thr-Gly-Leu-Arg (29), where the order of residues in parentheses was not determined. A Chou-Fasman analysis of this polypeptide yields the results shown in Table III. The degeneracy in the sequence of residues 7-9 and 10-11 did not alter the predicted structure. The most striking feature of the calculations is the prediction that the "active" cysteinyl residue should occur at the beginning of a β turn which separates two portions of β sheet structure. In such a position this sulfhydryl group could well be involved in conformational changes in the protein.

Kuntz (29) has observed that few β turns ever occur internally in the tertiary structure of proteins, a situation stemming from the hydrophilic nature of the amino acid side chains found in β turns. Conversion of the relatively hydrophilic thiol group in native creatine kinase to the more hydrophobic mixed disulfide in the CH₃S-blocked enzyme could perturb the β turn structure, and any spatially associated regions of the protein. Similarly, modification of the reactive sulfhydryl with a hydrophilic sulfhydryl reagent, e.g. iodoacetic acid, iodoacetamide, or S,S’-dithiobis(2-nitrobenzoic acid), would be expected to stabilize the β turn with hydrophilic side chains being solvated.

If one postulates that the active sulfhydryl group necessarily becomes buried during catalysis (i.e. less solvated as a result of the conformational change associated with substrate binding), then chemical modifications resulting in a more hydrophobic nature for the modified cysteinyl side chain could prevent this burying and obliterate catalysis. Conversely, if chemical modifications lead to a more hydrophobic nature for this group, this hypothetical conformational change could still occur. Evidence linking conformational changes to substrate binding has come from many sources, including susceptibility to tryptic cleavage (30), reactivity of the active site sulfhydryl.
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(1), magnetic resonance studies (1, 31, 32), intrinsic fluorescence (33), dye-binding studies (21), and immunological studies (34). While the role for the active cysteinyl residue in mediating conformational changes suggested above is purely speculative, it is nonetheless consistent with all previous observations of structural changes and kinetic changes.

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Note Added in Proof—Very recently, Price and Hunter (35) have reported that in the presence of creatine, MgADP, and nitrate the active sulphydryls of the two subunits of rabbit muscle creatine kinase react with thiol blocking reagents at different rates from each other, behavior consistent with a conformational change in the second subunit brought about by modification of a thiol group of the first subunit.

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