Spectrochemical and Ligand-Binding Studies of an Active Mercurinitrophenol-Labeled Creatine Kinase†

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ABSTRACT: The formation of several complexes between an active creatine kinase (muscle-type) labeled with 2-mercuri-4-nitrophenol and substrates and anions, singly or in combination, elicited different nitrophenol spectral changes. Blue shifts at different wavelength (between 405 and 426 nm) were observed in all but two spectral changes. Although the Mg^{2+} cofactor did not produce a change, its presence in the ternary E-MgADP complex caused a perturbation different from that generated by ADP alone. Creatine does not also induce a change, but conferred pronounced effect upon binding to E-MgADP and E-MgADP-nitrate complexes. In a series of complexes that ultimately make up the transition state analogue, E, E-MgADP, E-MgADP-nitrate or E-MgADP-creatine, and E-MgADP-nitrate-creatine, the spectral shifts, both in the visible and ultraviolet region, were different. These varied spectral changes and the finding that the rates of reaction of 2 equiv of mercurinitrophenol with the native enzyme in the same series were reduced in the order, 0-, 2-, 3- or 13-, and 200-fold, respectively [Quiocho, F. A., and Olson, J. O. (1974), *J. Biol. Chem. 249,* 5885], are manifestations of the various conformational states of creatine kinase. Anion inhibitors—chloride, nitrate, or sulfate—caused hypochromic shifts at 408, 406, or 400 nm, respectively, whereas acetate, an activator, brought about a hyperchromic shift at 415 nm. Pyrophosphate inhibitor caused a 439-nm red shift. Further reaction of the nitrophenol-labeled enzyme with mercurinitrophenol, iodoacetamide, or 2 equiv of methylmethanethiosulfonate resulted in the modification of another pair of cysteine residues and the loss of activity. The loss of activity occurred concomitant with structural rearrangement that was monitored by the bound chromophore. Further evidence, including the peptide mapping experiment, indicates that the chromophoric probe (at 2 equiv) reacted with 1 cysteine per subunit different from the essential thiol. Therefore, the structural change that is characteristic of each E-S complex and uniquely monitored by the chromophoric probe presumably occurs elsewhere than at the active site region.

A pair of cysteine residues of the muscle-type creatine kinase, or 1 thiol per subunit, can be reacted with organomercurials such as 2-chloromercuri-4-nitrophenol (Quiocho and Thomson, 1973) or methylmercury chloride (Smith et al., 1975) without the loss of enzymatic activity. The 2-chloromercuri-4-nitrophenol, an environment-sensitive spectral probe, can be further utilized to assess the conformational states of creatine kinase in various E-S complexes. For instance, although the stoichiometry of the reaction and enzymatic activity were unaffected, the rates of reaction of 2 equiv of chromophore with a variety of enzyme-substrate complexes were found to be reduced anywhere between 2- to 200-fold relative to the reaction of the native enzyme (Quiocho and Olson, 1974). The postulated transition state analogue E-MgADP-nitrate-creatine caused the most severe reduction in rates. These rate differences were attributed to varied changes in the accessibility of the mercury-specific thiol group. In this communication we wish to report that the spectrum of the nitrophenol incorporated to creatine kinase (without loss of activity) can be utilized not only to further assess the complexed states of creatine kinase, but also to monitor structural changes resulting from inactivation by other thiol-specific reagents. We wish also to present evidence, including "peptide mapping" experiment, which indicates that the organomercurial reacts with a thiol group at a site distinct from the active site region. Therefore, the structural change that is characteristic of each enzyme-substrate complex and uniquely monitored by the "reporter group" presumably occurs at a locus different from the active site.

Materials and Methods

Materials. New Hampshire chicks (Normal, line 200) were obtained from the Department of Avian Sciences, University of California at Davis, and allowed to grow for approximately 6 months prior to isolation of creatine kinase. Iodo[1-^{14}C]-acetamide and iodo[2-^{14}C]acetic acid were obtained from Amersham/Searle. 2-Chloromercuri-4-nitrophenol (MNP)[1] was a gift of Dr. C. H. McMurray and also obtained from Eastman Kodak and recrystallized. Methylmethanethiosulfonate (MMTS) was synthesized very kindly by Shelly Hayes in this laboratory according to the procedure of Smith et al. (1975). All other chemicals were of reagent grade and used without further purification. Water was deionized and subsequently glass distilled.

Enzyme Purification and Assay. Creatine kinase was isolated from the breast muscle of New Hampshire chickens (line 200) by the procedure originally developed by Kuby et al. (1954) for the purification from rabbit muscle and modified by Roy et al. (1970) for the enzyme from chicken breast muscle.

All five preparations of the enzyme gave a single band on sodium dodecyl sulfate gel electrophoresis, a single elution peak on Sephadex G-100 column chromatography, an amino acid analysis consistent with published value (Roy, 1974), and a

† From the Department of Biochemistry, Rice University, Houston, Texas 77001. *Received December 17, 1976; revised manuscript received May 31, 1977.* This investigation was supported by a National Institutes of Health Grant (AM-17008) and a grant from the Robert A. Welch Foundation (C-581).

[1] Abbreviations used: Bicine, *N,N*-bis(2-hydroxyethyl)glycine; TEA, triethanolamine; MMTS, methylmethanethiosulfonate; MNP, 2-mercuri-4-nitrophenol; E(MNP), creatine kinase labeled with 2 equiv of MNP; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance.

specific activity of 130 μmol of H^+ released min^{-1} (mg of enzyme)$^{-1}$. Enzyme preparations were stored under N_2 in 20 mM Tris-HCl (pH 8.00) buffer which contained dithiothreitol (5 mM). Prior to use, the enzyme was exhaustively dialyzed with the appropriate buffer solution in order to remove the dithiothreitol.

Protein concentration was obtained spectrophotometrically at 280 nm, with an absorption coefficient of 0.89 (mg/mL)$^{-1}$ cm^{-1} (Noda et al., 1954). The molecular weight of 80 000 for creatine kinase (Noda et al., 1954; Watts, 1970) was used to determine molar concentrations.

The pH-stat method (Radiometer pH-stat system) was routinely used to measure enzyme activity at pH 9.00, 30 °C (Mahowald et al., 1962). The standard 3-mL assay solution consisted of 4 mM ATP, 5 mM magnesium acetate, 40 mM creatine, 0.1 M sodium acetate, and bovine serum albumin (1 mg/mL). Assays were done in a nitrogen atmosphere.

Spectrophotometric Measurements. All measurements were carried out with the use of a Cary 118 spectrophotometer with the gain set at 0.25 and auto slit. The temperature was maintained at 30 °C.

For difference spectrum measurements, matched quartz sectored cells with 87.6-mm total light path were used. For each scan, the pen was balanced at 550 nm, where all solutions were transparent. An initial baseline scan was obtained when equal volumes of the labeled enzyme were placed in the front section and identical volumes of buffer in the back section of both cells. Each difference spectrum was then generated by adding (with a Hamilton microsyringe) equal volumes of perturbant to the front of the test cell and the back of the reference cell. The dilution effect was corrected for by adding identical volumes of buffer to the other compartments of the respective cells. A baseline scan was again obtained after mixing of the contents of both cells. Solutions were buffered at pH 8.00 ± 0.01 with 100 mM Bicine–NaOH.

Creatine Kinase Labeled with Mercurinitrophenol. E(MNP) was prepared by incubating creatine kinase (about 4 mg/mL), previously dialyzed exhaustively against 100 mM TEA-HCl (pH 7.90), with 2 equiv of MNP at 4 °C for 1 h. The incubation was then dialyzed exhaustively against 0.1 M Bicine (pH 8.00) and assayed for activity.

"*Peptide Mapping*". The following chemically modified creatine kinase was subjected to "fingerprinting" experiments. (Unless otherwise noted all modifications were carried out in 0.1 M Bicine buffer (pH 8.00) using an enzyme concentration of 2.5 mg/mL. Following the modification and prior to tryptic digestion, the protein was dialyzed against 0.1 M NH_4HCO_3.) (1) Creatine kinase modified at all cysteine residues with iodo[1-^{14}C]acetamide or iodo[2-^{14}C]acetic acid. The enzyme in the presence of 8 M urea was reacted with 20 mol excess of the reagent for 2 h at room temperature. (2) Native enzyme ^{14}C-labeled at the so-called "essential thiol". The enzyme was reacted with 100 mol excess of iodo[1-^{14}C]acetamide for 1 h at room temperature. After dialysis against 0.1 M Bicine (pH 8.00) in order to remove excess reagent, the enzyme (in 8 M urea) was reacted with 20 mol excess of cold iodoacetamide for 2 h at room temperature. (3) Creatine kinase labeled with 2 equiv of MNP. (4) Creatine kinase labeled with 2 equiv of MNP and subsequently ^{14}C labeled. Preparation was identical with that in 2 except that the enzyme was labeled with 2 mol MNP/mol of enzyme prior to reaction with iodo[1-^{14}C]acetamide. (5) Creatine kinase modified with iodo[1-^{14}C]acetamide at the MNP-specific thiol residues. Enzyme previously labeled with 2 equiv of MNP was reacted with 0.1 M iodoacetamide in the presence of 8 M urea at room temperature for 2 h and dialyzed vs. 0.1 M Bicine (pH 9.00). The pH of the dialyzate may have to be raised to 9.4 in order to keep the protein in solution. The alkylated MNP-labeled creatine kinase was then treated with 5 mM 2-mercaptoethanol for 30 min in order to displace the MNP and then dialyzed overnight vs. 0.1 M Bicine–5 mM 2-mercaptoethanol (pH 9.00). The excess mercaptoethanol was removed by dialysis vs. 0.1 M Bicine (pH 8.00) under N_2. The alkylated enzyme which is rid of MNP was finally treated for 2 h with 20 mol excess of iodo[1-^{14}C]-acetamide in 8 M urea. (6) Creatine kinase labeled at the "essential thiol" with cold iodoacetamide and at all other thiols with iodo[2-^{14}C]acetic acid. Creatine kinase was treated with 100-fold mol excess of iodoacetamide at room temperature for 1 h and dialyzed against buffer to remove unreacted reagent. The protein in 8 M urea was reacted with 20 mol excess of iodo[2-^{14}C]acetic acid for 2 h at room temperature.

Trypsin digestion was done at a trypsin:creatine kinase ratio of 1:50 (w/w) for 2.5 h at 30 °C. The digest was then lyophilized.

For two-dimensional peptide maps, a tryptic digest was spotted on a Whatman No. 3MM paper, 57 × 46 cm, with approximately three-fourths of the protein material in a 5-cm band and the remainder of the solution in a 1-cm band near one edge of the paper. High voltage electrophoresis was carried out at pH 6.5 in pyridine–acetic acid buffer, using a modified apparatus originally described by Michl (1951). Following electrophoresis, a lengthwise strip containing the 1-cm band was cut off for use as a guide strip. The remaining map was then subjected to ascending chromatography at right angle for 20 h in a solvent system of pyridine–1-butanol–acetic acid–water (60:90:18:72, by volume).

Peptides were detected with a spray consisting of 0.02% ninhydrin in 95% ethanol. Radioactively labeled peptides were first located on the guide strip by cutting the strip into 1-cm segments and counting in a scintillation counter. Areas of the two-dimensional map where radioactivity would be expected were then noted and all ninhydrin-staining spots in these areas cut out and counted.

Results

Inactivation of E(MNP) with Thiol-Specific Reagents. Iodoacetamide and methylmethanethiosulfonate (MMTS) have been shown to inactivate native creatine kinase as a result of the reaction of what has been considered to be a pair of "essential" cysteine residues (Watts and Rabin, 1962; O'Sullivan et al., 1966; Smith and Kenyon, 1974; Smith et al., 1975). Whereas the reaction of iodoacetamide causes the complete loss of activity, the reaction of 2 molecules of MMTS per enzyme molecule still leaves apparently about 18% residual activity. Since creatine kinase can be labeled with 2 equiv of MNP with an apparent preservation of activity (Quiocho and Thomson, 1974), presumably another pair of thiols can be modified by MMTS or iodoacetamide. Utilizing the bound nitrophenol spectrum, it was possible to follow the reaction of twofold mol excess of MMTS with E(MNP) (Figure 1). The rate observed for the spectral change at 445 nm for the reaction of E(MNP) with methylmethanethiosulfonate or iodoacetamide, and the rate of inactivation by 2 equiv of MMTS are shown in Figure 2. The spectral change associated with the reaction of methylmethanethiosulfonate or iodoacetamide occurs with an apparent first-order kinetic (Figure 2, insert). However, the loss of activity associated with the reaction of CH_3S– proceeds with a sharp initial drop to be followed by a very gradual loss of activity to a final value of about 8% residual activity. The inhibition of the native enzyme by the MMTS was observed to be rapid and not biphasic. In the reaction of the MMTS with the native or MNP-labeled enzyme, we have

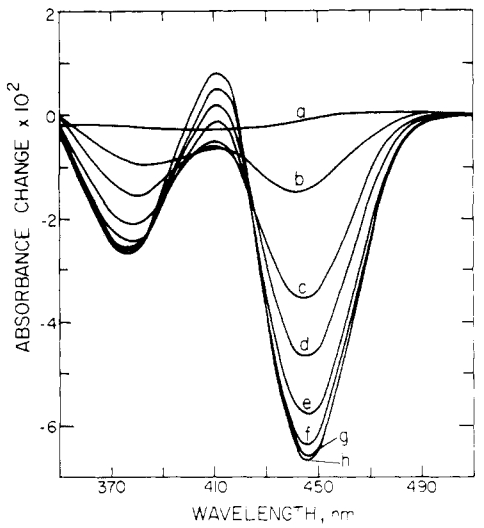

FIGURE 1: Difference nitrophenol spectral change produced by the reaction of 2 equiv of MMTS with E(MNP) as a function of time. (a) Baseline scan: sample and reference cells (1-cm path length) contained 3.2 mg of E(MNP) in 1.96 mL of 100 mM Bicine, pH 7.8. Curves b, c, d, e, f, and g are scans after addition of 40 μL of 2 mM MMTS to sample cell and an equivalent amount of water to reference and further incubation for 1, 6, 11, 30, 45, and 61 min, respectively.

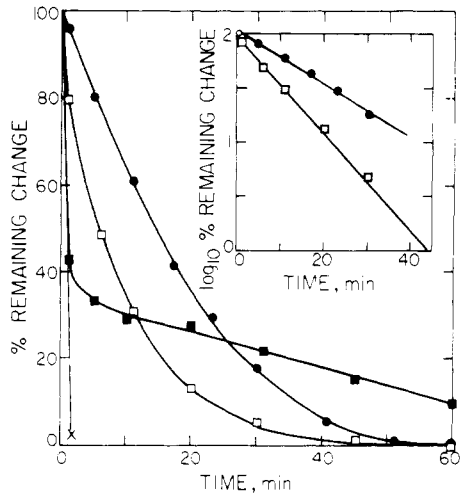

FIGURE 2: Rate of reaction of E(MNP) with thiol reagents. The 50-fold excess iodoacetamide: (a, ●) rate of spectral change at 444 nm. Similar incubation as described in Figure 1 except that 50 mol excess of iodoacetamide was used. Percent spectral change remaining = $A_{final} - A_t / A_{final} \times 100$. The 2-fold excess MMTS: (b, □) rate of spectral change at 446 nm, data from Figure 1; (c, ■) inactivation of 50 μM E(MNP) by 100 μM MMTS, expressed as percent remaining activity; (d, X) inactivation of 50 μM native creatine kinase with 100 μM MMTS. (Insert) Logarithmic plots of bound MNP spectral change with time showing first-order kinetics: (□) reaction of MMTS; (●) reaction of iodoacetamide.

generally observed rather low residual activities of anywhere in the range of 0–8%.

We have shown previously that, depending on the buffer-anion used, the reaction of 2 equiv of MNP with the enzyme produced spectral changes that were different. In TEA-HCl (pH 8.00) buffer, the difference spectrum showed a pronounced negative absorbance change at 390 nm, whereas in Bicine-NaOH (pH 8.00) the reaction resulted in a large hyperchromic shift at about 430 nm (Quiocho and Olson, 1974). The dissimilar spectral change was attributed to the presence of the chloride anion inhibitor in TEA-HCl buffer. The second-order rate constants for the reaction of 2 equiv of MNP in both buffers were found to be about 10^7 M^{-1} s^{-1}. Here we

TABLE I: Mercury Analysis of Creatine Kinase Reacted with 2-Chloromercuri-4-nitrophenol.[a]

Enzyme, buffer pH 8.00	Mole excess of MNP incubated[b] 2	6	8
	No. of Hg bound/enzyme molecule		
Native, TEA-HCl	1.9 ± 0.2[c]		
Bicine-NaOH	1.8 ± 0.1[c]		
TEA-HCl			4.0 ± 0.1[c]
TEA-HCl			3.8 ± 0.1[d]
Bicine-NaOH			3.9 ± 0.2[c]
E(MNP), TEA-HCl		3.9 ± 0.2[d]	

[a] Mercury analysis by atomic absorption spectrometry was accomplished with the use of a Norelco Unicam SP-90 A spectrophotometer or Instrumentation Lab Inc. Atomic Emission spectrophotometer 153. MNP was used to make a Hg standard curve. [b] Reactions of creatine kinase with the indicated mole excess of MNP were done at room temperature in the 100 mM buffer indicated. The enzyme concentrations used were between 8.3 and 9.5 mg/mL. [c] Reaction mixture was immediately chromatographed onto a Sephadex G-25 column prior to mercury analysis. [d] Reaction mixture further incubated for 1.5 h before being chromatographed.

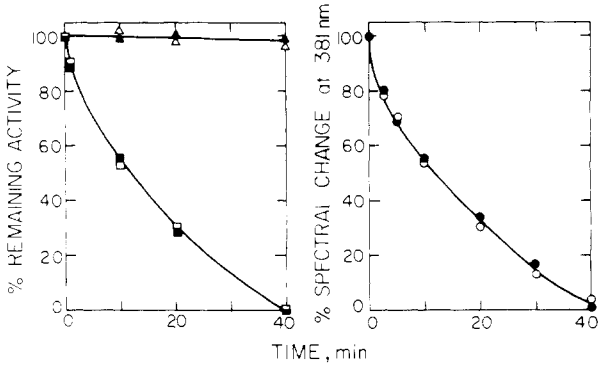

FIGURE 3: The effect of excess MNP on the nitrophenol-bound spectrum and activity of creatine kinase. All incubations were done in 100 mM TEA-HCl (pH 8.00). (a, ●) Difference spectral change at 381 nm resulting from incubation of 25 μM creatine kinase with 200 μM MNP. Percent spectral change = $A_{max}/A_{time} \times 100$. (b, ○) Difference spectral change following the reaction of 25 μM E(MNP) with 150 μM MNP. (c, ■) Inactivation of 25 μM E(MNP) with 150 μM MNP. At the indicated time, 1 μL (2 μg of enzyme) of incubation mixture was removed and added directly to a 3-mL standard assay mixture. (d, □) Inactivation of 25 μM creatine kinase with 200 μM MNP. Other conditions as in c. (e, △) With 25 μM enzyme plus 50 μM MNP. (f, ▲) Control, incubation of native enzyme. In curves c to f, 100% activity corresponds to about 130 μmol of H^+ min^{-1} (mg of enzyme)$^{-1}$.

show by atomic absorption analysis that the enzyme incubated with 2 equiv of MNP in both buffers and subjected to chromatography onto Sephadex G-25 contained 2 atoms of mercury per enzyme molecule (Table I). Furthermore, addition of 6 mol excess of MNP to a solution of E(MNP) further produced two different spectral changes. The *first* is an instantaneous change in which the difference spectrum maximum at 430 nm observed upon association of 2 equiv of MNP in Bicine buffer was shifted to 420 nm (the minimum in TEA-HCl at 390 nm to 384). Analysis of the rate of the instantaneous spectral change by stopped-flow technique indicated a first-order kinetic with $t_{1/2}$ of 11 s. (This rate is 700 times slower than the rate of reaction observed with 2 equiv of MNP.)[2] Further incubation of the mixture of E(MNP) and

[2] At high MNP concentration, the rate of change previously attributed to the reaction of 2 equiv of MNP with the native enzyme (Quiocho and Olson, 1974) was as expected too fast to be observed in the present measurement.

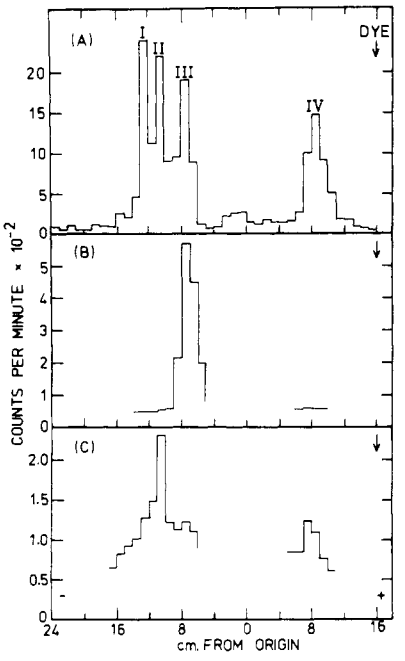

FIGURE 4: Radioactive histograms of strips resulting from electrophoresis of tryptic digests of ^{14}C-labeled creatine kinase (see Materials and Methods). (A) Creatine kinase ^{14}C-labeled at all cysteine (modified enzyme No. 1). (B) Creatine kinase ^{14}C-labeled alkylated at the "essential thiol" (No. 2). (C) Enzyme labeled with [^{14}C]iodoacetamide at the MNP-reactive thiol (No. 5). Radioactive peaks are labeled I, II, III, and IV.

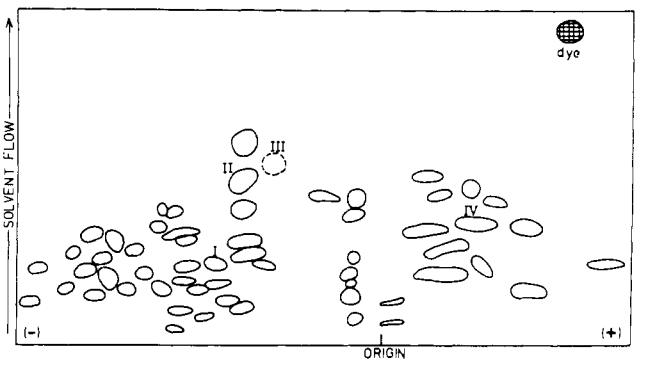

FIGURE 5: Tracing of "fingerprint" tryptic digest of chicken breast muscle creatine kinase modified at all cysteine residues with iodo[1-^{14}C]acetamide (modified enzyme No. 1, see Materials and Methods). Spots I, II, III, and IV are ^{14}C-labeled peptides. (Broken circle) Spot which stained faintly with ninhydrin.

6 equiv of MNP produced a *second,* much slower spectral change which is characterized by a further shift in absorbance maximum to shorter wavelength and changes in the magnitude of the extinction. Rates of the slow spectral change at 381 nm (TEA–HCl (pH 8.00)) and loss of activity are shown in Figure 3. The rate of the slow spectral change was the same regardless of whether 6 mol excess of MNP was added to E(MNP) (Figure 3, curve b) or whether 8 mol excess was mixed directly with the native enzyme (Figure 3, curve a). Furthermore, the slow spectral changes and loss of activity are first order with apparently similar $t_{1/2}$'s of about 10 min. The inhibited enzyme can be reactivated to its original full activity by reduction with 5 mM mercaptoethanol. The enzyme incubated with eightfold excess MNP for 2 min showed about the same amount of bound mercury as the 90-min incubation sample: about 4 mol per mol of enzyme (Table I). Apparently, prolonged incubation of the enzyme in the presence of excess mercurial does not lead to an increase in the number of thiols modified, although, as was shown in Figure 3, further slow spectral change simultaneous with loss of activity was observed.

"*Peptide Mapping*". The guide strips from electrophoreses of creatine kinase labeled with iodo[1-^{14}C]acetamide under denaturing conditions (modified enzyme No. 1, see Materials and Methods) consistently showed three slightly overlapping radioactive peaks (I, II, III) in the region of migration toward the cathode and a fourth, broader peak (IV) among the acidic peptides (Figure 4A). The number of observed radioactive peaks corresponds to the number expected on the basis of the cysteine content of chicken breast muscle creatine kinase—8 cysteines, or 4 thiols per subunit. Three ninhydrin-staining radioactive peptides corresponding to peaks I, II, and IV were located on two-dimensional peptide maps (Figure 5, for example). The fourth radioactive peptide (III), predicted on the basis of the guide strip, stained only very faintly.

The peptide containing the "essential thiol" was identified from maps of digest of ^{14}C-labeled native enzyme (modified enzyme No. 2) and ^{14}C-labeled enzyme previously modified with 2 equiv of MNP (modified enzyme No. 4). The essential thiol peptide corresponded to the site that stained very faintly with ninhydrin (peptide III, Figures 4B and 5). Amino acid analysis of the essential thiol peptide showed exactly those amino acids reported by Roy (1974) in his sequence analysis of the peptide containing the essential thiol in chicken breast muscle creatine kinase (Table II).

The peptide containing the MNP-specific thiol group was identified on maps as a purple-staining basic peptide migrating close to the essential thiol peptide (peptide II, Figures 4C and 5). The "MNP-peptide" was identified from tryptic digests of enzyme with the MNP-reactive thiol modified with iodo[^{14}C]acetamide (modified enzyme No. 5). Direct detection from electrophoreses of the MNP-containing peptide (modified enzyme No. 3 and 4) on the basis of its yellow color at basic pH basically agrees with this identification.

Results of amino acid analysis of spots identified on two-dimensional maps as the peptide containing the "essential thiol" and the peptide containing the MNP-specific thiol are shown in Table II. Unexpectedly, the spot which corresponds to the "MNP peptide" was found to contain both lysine and arginine residues. Two likely possibilities could account for this finding: first, that the peptide resulted from an unusual tryptic cleavage due to a sequence, e.g., a juxtaposition of lysine and arginine or, second, that the spot represented two cochromatographing peptides. The presence of considerably more arginine than lysine makes the second possibility seem more plausible. To the nearest whole number, if the spot were one peptide with 2 arginines and 1 lysine, then it would be expected to migrate much farther in the direction of the cathode during electrophoresis. If the spot represented two peptides, however, one of which was eluted in a somewhat better yield than the other, then both would have a net charge which would possibly make them cochromatograph. Peptide mapping of creatine kinase labeled with iodo[2-^{14}C]acetic acid at all but the essential thiol (modified enzyme No. 6) showed that the "MNP peptide" was now located among the neutral peptides. Unfortunately, the MNP peptide overlapped with other neutral peptides so that amino acid analysis was uninterpretable. However, analysis of the peptide that originally cochromatographed with the MNP peptide makes it possible to qualitatively predict an amino acid composition for the peptide containing the MNP-reactive thiol (Table II).

Ligand Perturbations of the Enzyme-Bound Nitrophenol Spectrum. Although creatine kinase can be labeled with 2 equiv of MNP without the loss of activity, the bound nitrophenol spectrum can be perturbed by the formation of en-

TABLE II: Amino Acid Composition of Peptides from Tryptic Digests of Creatine Kinase.

Amino acid	Essential thiol peptide		MNP Spot		
	This work[a]	From Roy (1974)	Total anal.[a,b]	Contaminant[c]	MNP-peptide[d] (pred)
Lys			1.0		1.0
Arg	0.8	1	1.6	1.6	
S-CM-Cys	1.0	1	1.0		1.0
Asp	1.1	1			
Thr	1.7	2			
Ser	1.4	1	2.9	3.2	
Pro	1.4	1			
Gly	2.3	2	1.5	1.6	
Val			2.8	1.6	1.2
Ile	0.9	1			
Leu	2.9	3	2.9	1.6	1.3
Tyr			1.4		1.4
Phe			1.0		1.0

[a] Based on cysteine, measured as *S*-carboxymethylcysteine. [b] Represents both MNP-peptide and a cochromatographing or contaminant peptide. [c] Based on arginine, assuming arginine content of total analysis due entirely to contaminant peptide. [d] Based on the difference between total analysis and contaminant.

zyme–substrate complexes. In this study, we have extended the earlier, very limited and preliminary investigations of the effect of substrates on the nitrophenol spectrum (Quiocho and Thomson, 1973). We have systematically studied the various spectral changes generated during successive ligand additions in the formation of the enzyme–MgADP–nitrate–creatine complex, a transition state analogue complex (Milner-White and Watts, 1971), and the effect of anions and other abortive and productive complexes on the nitrophenol spectrum. Furthermore, whereas the previous study was conducted in triethanolamine hydrochloride buffer (pH 9.00), the present experiments were performed in 100 mM Bicine–NaOH (pH 8.00), thus avoiding any effects of chloride anion. Chloride anion has been shown to be a potent inhibitor of the enzyme (Milner-White and Watts, 1971).

The enzyme-bound nitrophenol spectral changes generated by the formation of various complexes are shown in Figures 6 and 7. (Typical baseline scans are shown in Figure 6I.) It is apparent from these spectra that various ligands and some combinations thereof elicited different spectral changes. (These ligands did not affect the spectrum of 2-mercuri-4-nitrophenol reacted with 2-mercaptoethanol.) The shapes and locations of wavelength peaks, indicators of changes in the microenvironment of the chromophore, are different. These changes are particularly exemplified by the diverse changes in the spectrum arising from the formation of a series of complexes which ultimately make up the E–MgADP–nitrate–creatine complex (Figure 6II). (The concentrations of ligands used in the spectral measurements are: E(MNP), 16.7 μM; Mg^{2+}, 8.33 mM; ADP, 1.25 mM; nitrate, 83.3 mM; and creatine, 26.7 mM.)[3] This complex has been suggested to be an analogue of the transition state (Milner-White and Watts, 1971; Reed and Cohn, 1972). According to this suggestion, the planar nitrate binds at a site of the vacant γ-phosphoryl group, between creatine and MgADP. Spectral changes over the wavelengths scanned were not observed when creatine (Figure 6a) or magnesium acetate (Figure 6c) was mixed with the labeled enzyme. However, all other combinations generated characteristic blue shifts at anywhere between 426 and 405 nm. A blue shift within this region is usually attributed to the protonated form of the *p*-nitrophenol moiety. Despite these shifts, however, fewer trends or compensatory changes were apparent in the region below 400 nm in the visible spectrum, where a red shift would also be ascribed to protonated *p*-nitrophenolic group. However, nitrate by itself (Figure 6b) or in association with the ternary E(MNP)–MgADP complex and with the quaternary E(MNP)–MgADP–creatine complex produced changes with positive peaks at the region between 330 and 340 nm (Figure 6f and 6g). In the absence of nitrate, however, these positive peaks were, in the case of the ternary complex, absent, or in the quaternary complex, flattened. Moreover, the wavelength minimum observed with the ternary complex (426 nm) and quaternary complex (424 nm) shifted to 405 nm and 417 nm, respectively, upon the addition of nitrate to each complex.

The nitrophenol-labeled enzyme was further utilized to probe the effect of various anions (Figure 7). Of those anions tested, all but acetate, which apparently enhances enzyme activity, have been shown to inhibit enzyme activity (Nihei et al., 1961; James and Morrison, 1966; Milner-White and Watts, 1971). Of notable finding is the vast contrast between the effects of chloride, an inhibitor, and acetate, an activator. KCl (83.3 mM final concentration) produced a hypochromic shift at 408 nm (Figure 7a), whereas 83.3 mM sodium acetate elicited a hyperchromic shift at 415 nm (Figure 7e).

Utilizing the nitrophenol spectral change associated with the binding of ligands to E(MNP), it is possible to determine the equilibrium constant of the binding, for example, of ADP. This determination is easily possible provided that the spectral changes occur at a reasonably fast rate. This proviso is apparently the case as indicated by the finding that the rate of bound nitrophenol spectral change elicited by ADP was too fast to be observed by stopped-flow rapid mixing technique (Quiocho and Olson, unpublished result). The result of titration of the chromophore-labeled, active creatine kinase with ADP showed a K_d of 0.17 mM for the E(MNP)–ADP complex.

Discussion

This study clearly shows that the nitrophenol incorporated to creatine kinase without loss of activity can be easily utilized

[3] With the exception of creatine or Mg^{2+} which binds very slightly (see also Kuby et al., 1962) and elicits no spectral change, the concentration of ADP, MgADP, or nitrate was at saturating conditions. Furthermore, it has been shown that nitrate profoundly lowers the K_i value for creatine and MgADP (Milner-White and Watts, 1971) and that the K_d of creatine in E–Mn–ADP and E–Mn–nitrate–creatine complexes is 3 and 0.08 mM, respectively (McLaughlin et al., 1972).

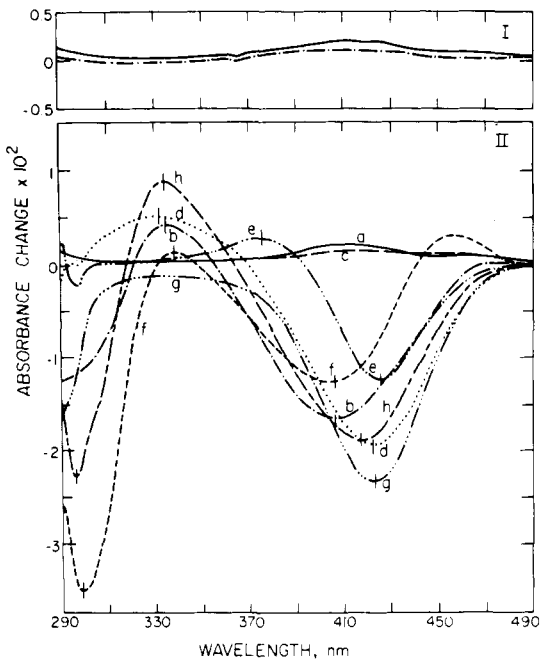

FIGURE 6: Difference spectra produced by addition of substrates and/or anion to creatine kinase labeled with 2 equiv of MNP. Buffer used: 100 mM Bicine–NaOH (pH 8.00). (See Materials and Methods for procedure.) (I) Typical baseline scans. Initial baseline (—); front section of test and reference cells contained 1.17 mL (17.1 μM) of E(MNP); back section contained equal volumes of buffer. A final baseline scan (—●—); concentrations after mixing content of cells—8.33 μM E(MNP), 0.625 mM ADP. (II) Addition of ligands to E(MNP). Concentration of ligands: 8.33 mM $Mg(OAc)_2$, 1.25 mM ADP, 83.3 mM $NaNO_3$, 26.7 mM creatine. Perturbants in each curve: (a) creatine; (b) $NaNO_3$; (c) $Mg(OAc)_2$; (d) ADP; (e) MgADP; (f) MgADP, nitrate; (g) MgADP, creatine; (h) MgADP, nitrate, creatine.

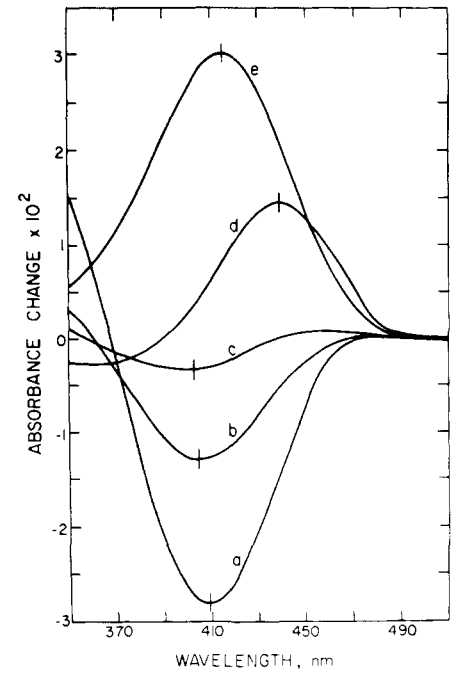

FIGURE 7: Effect of anions on E(MNP) nitrophenol spectrum. Concentration of anions: (a) 83.3 mM KCl; (b) 83.3 mM $NaNO_3$; (c) 83.3 mM Na_2SO_4; (d) 41.7 mM sodium pyrophosphate; (e) 83.3 mM Na(OAc).

to differentiate the various liganded or complexed states of the enzyme. Quiocho and Olson (1974) have also shown that the conformational states of similar complexes can be further assessed on the basis of the differences in rates of reaction of the organomercurial with E–S complexes. The structural change that distinguishes each complex and is uniquely reflected in both the rate of reaction and the bound nitrophenol spectrum presumably occurs at a site distinct from, and as a consequence of the binding of substrates at, the active site region. This supposition is primarily based on the finding that the reaction of the mercurinitrophenol does not result in the loss of enzymatic activity and in the modification of the so-called "essential thiol". Heretofore, other techniques have provided assessments of the active site region directly during formation of some complexes (see review by Watts, 1973). It is also interesting to note that the various complexes elicited different spectral changes around 295 nm (Figure 6), presumably due in part to the changes in the intrinsic tryptophan absorption (Wetlaufer, 1963; Edelhoch, 1967).

The lack of spectral perturbation by Mg^{2+} is consistent with the finding by Kuby et al. (1962) that the metal activator alone does not bind to the enzyme. However, the formation of the ternary E(MNP)–MgADP complex produced a nitrophenol spectral change that differed with the change induced by ADP alone. In addition, ADP appears to exert a far greater effect than the true MgADP substrate. This is reflected by the finding that the rate of reaction of 2 equiv of chromophore with the binary E–ADP complex decreased by sevenfold, whereas the ternary E–MgADP complex decreased by only twofold relative to the rate of the unliganded native enzyme (Quiocho and Olson, 1974). A similar decrease in rates was also observed with E–ATP and E–MgATP complexes, seven- and fourfold, respectively. These results that show differences between nucleotides and their magnesium complexes indicate that the metal activator exerts appropriate changes in the structural organization of the E–Mg–nucleotide complexes. The possibility that the metal in these complexes may have at least one ligand to the protein could account for the pronounced effect of Mg^{2+}.

The different effects of nucleotides as compared with their corresponding magnesium complex have been observed previously, for instance, against inhibitions of the enzyme by thiol-specific alkylating reagents (Watts and Rabin, 1962; O'Sullivan et al., 1966; O'Sullivan and Cohn, 1966) and toward a spin-label bound to the essential thiol (Taylor et al., 1971). O'Sullivan et al. (1966) showed that the so-called essential thiol residue of creatine kinase can be protected by ADP against the slow reaction of iodoacetate (in large excess) while MgADP accelerated the inhibiting reaction. Moreover, correlation of the reactivity of the essential thiol and proton magnetic resonance spectrum with the activity of the enzyme toward a number of different Mn–nucleotide substrates indicated the existence of at least five differing degrees of conformational changes induced at the active site of creatine kinase (O'Sullivan and Cohn, 1966).

The finding that the dissociation constant of the complex between the nitrophenol-labeled active creatine kinase and ADP is within the range of constants (0.1–0.3 mM) determined for the enzyme–ADP complex (Nihei et al., 1961; Kuby et al., 1962; Morrison and O'Sullivan, 1965) suggests that the nucleotide binding site is not altered by the incorporation of the chromophore.

The diversified effect of anions can be classified as follows. First, some anions produced hypochromic shifts. Coincidentally, the anions that caused these shifts—chloride, nitrate, and sulfate—have been shown to inhibit enzymatic activity (Milner-White and Watts, 1971; Watts, 1973). Moreover, the spectral shifts were located progressively toward shorter wavelength in the order that the anion inhibitors are listed.

Second, acetate brought about a red shift. This vastly different shift may be related to the observation that acetate activates enzymatic activity. The unique influence of acetate by itself and when it combines with E(MNP)–MgADP–creatine suggests that perturbations elicited by this activator emanate from a site different from the anion inhibitor binding site. And third, pyrophosphate produced a red shift at a much longer wavelength than that observed in all the other spectral changes. Pyrophosphate has been shown to be a competitive inhibitor of both the forward and reverse reactions presumably because it binds at the phosphate binding site which is common to both MgATP and creatine phosphate (James and Morrison, 1966; Nihei et al., 1961).

The absence of an effect by creatine on both the bound nitrophenol spectrum and the rate of reaction is in accord with previous observations that creatine alone fails to affect either the EPR spectrum or the water proton relaxation rate of a spin label attached to the "essential" thiol of creatine kinase (Taylor et al., 1971) and to produce any relaxation effect in a temperature jump measurement (Hammes and Hurst, 1969). However, in combination with other substrates, creatine exerts some influences. For instance, the difference wavelength minimum observed with the E(MNP)–MgADP–nitrate complex became, upon addition of creatine, more negative and shifted to longer wavelength. Furthermore, when creatine combines with the complex E–MgADP or E–MgADP–nitrate, the rate of reaction of the chromophore was reduced by about 13- or 200-fold, respectively, relative to that observed with the native enzyme. In contrast, these complexes in the absence of creatine reduced the rates to only about half that with the native enzyme.

The formation of the E–MgADP–nitrate–creatine complex is not only accompanied by a unique bound nitrophenol spectrum, but caused the most pronounced diminution in the rate of the reaction of the mercurial, as much as 200-fold (Quiocho and Olson, 1974). Not even the substitution of the nitrate in the complex by acetate or the incubation of the native enzyme with 2 mM MgATP and 20 mM creatine, substrates of the forward reaction, causes such a reduction in the rate. Although substitution of acetate for nitrate in the analogue complex produced a different spectral change (Laue and Quiocho, unpublished data), the reaction of the mercurial with E–MgADP–creatine–acetate caused only a 15-fold reduction in the rate (Quiocho and Olson, 1974). Likewise, the second-order rate in the presence of substrates for the forward reaction at pH 8.00 is 5.2×10^5 M^{-1} s^{-1}, only about 4-fold lower than the rate observed with the unliganded enzyme.

Studies that took cognizance of the inhibitory effect of anions, notably the enzyme kinetic and iodoacetamide inhibition studies of Milner-White and Watts (1971), have suggested that the anion-stabilized E–MgADP–nitrate–creatine complex may be an analogue of the transition state. Furthermore, these studies together with those of Cohn and co-workers (Cohn, 1970; Reed and Cohn, 1972), utilizing magnetic resonance technique, have shown that the formation of this analogue complex is accompanied by a substantial conformational change. The pronounced rate reduction and spectral change monitored by the nitrophenol spectral probe appear to reflect such a conformational change in the transition state analogue complex. Furthermore, in the series of complexes that ultimately make up the analogue complex—E, E–MgADP, E–MgADP–nitrate or E–MgADP–creatine, and E–MgADP–nitrate–creatine—the rates of reaction of the organomercurial decreased as follows: 0-, 2-, 3- or 13-, and 200-fold, respectively. The formation of these complexes, where the enzyme was labeled with 2 equiv of MNP, was further characterized by the production of varied nitrophenol spectral changes. These correlative results indicate that each ligand exerts appropriate changes and that, in the series, the enzyme undergoes a graded structural change. The changes that are monitored by the chromophore are presumably localized at a site distinct from the active site region and not necessarily indicative of gross structural rearrangement. Changes to varying degrees of nonpolarity of the bound nitrophenol environment could account for the blue shifts at different wavelengths (between 405 and 426 nm) observed in all but two of the spectral changes. In addition, these spectral (or conformational) changes were too fast to be observed by stopped-flow rapid mixing technique.

Chemical modification studies have indicated that a thiol residue/subunit of creatine kinase can be modified by a variety of other thiol-specific reagents with concomitant loss of activity (Watts et al., 1961; Mahowald et al., 1962; O'Sullivan et al., 1966; Okabe et al., 1970; Roy et al., 1970; Brown and Cunningham, 1970; Milner-White and Watts, 1971; Roustan et al., 1973). What appears to be an "essential thiol" has been shown in all isoenzymes of creatine kinase and enzymes isolated from various mammalian sources (Watts, 1973), including the one currently under study (Roy et al., 1970). More recently, however, evidence has indicated the nonessentiality of the active sulfhydryl group in creatine kinase (muscle-type) (Smith and Kenyon, 1974; Smith et al., 1975; Der Terrossian and Kassab, 1976). On the other hand, our studies suggest the existence of another pair of thiols which can be reacted selectively with organomercurial at a site where the bound chromophore can undergo varied environmental changes upon formation of various E(MNP)–S complexes. In addition to the finding that 2 equiv of mercurial do not inactivate the enzyme while other thiol-specific reagents do, other observations support this suggestion. For instance, the stoichiometry of the reaction and enzyme activity are unaffected when 2 equiv of the chromophore react with various enzyme–substrate complexes, in particular with the transition-state analogue complex of enzyme–MgADP–nitrate–creatine (Quiocho and Thomson, 1973; Quiocho and Olson, 1974). In contrast, the formation of the analogue complex protects the enzyme from inhibition by other thiol reagents (Watts, 1973; Milner-White and Watts, 1971; O'Sullivan et al., 1966). The creatine kinase previously reacted with 2 mol of probe can also be inactivated completely by reaction of methylmethanethiosulfonate or iodoacetamide, presumably as a result of the reaction of the essential thiol. Spectral study (Quiocho and Thomson, 1973) and "peptide mapping" experiment indicate that the mercurinitrophenol bound to the enzyme was apparently not displaced during the reaction of iodoacetamide. Further evidence for the existence of two different reactive pairs of thiols in the native enzyme has come from the observation that the incubation of 8 mol excess of MNP with the native enzyme or of 6 mol of MNP with E(MNP) leads to the reaction of another pair of thiols followed by a gradual loss of activity (Figure 3). Analyses of the amount of reacted mercury in both incubation mixtures yielded similar results—about 4 MNP bound per molecule of enzyme (Table I). Thus, a second pair of thiols, which may be identical with the so-called essential thiols, were modified in the presence of excess chromophore. Moreover, the fact that the reaction of 2 equiv of MNP with a pair of thiols is about 700-fold faster than with the other so-called essential pair (at 8 equiv of MNP) and that the resulting bound nitrophenol spectral changes are different must be related to differences in the microenvironment of the two pairs of sulfhydryl groups (Quiocho, unpublished data).

"Peptide mapping" experiments of tryptic digests of various

chemically modified creatine kinases have further provided conclusive evidence that the peptide which contains the MNP-specific cysteine residue is different from the peptide which contains the purported essential thiol residue.

Inactivation of enzymes resulting from chemical modification of specific and, by virtue of location, unusually reactive group(s) is generally considered to be due to the steric presence of a chemical adduct at or near the active site region. Conformational changes accompanying chemical modifications have often also been postulated to occur. However, such changes are often difficult to detect particularly if they are localized. Apparently, in the case of the chromophore-labeled active creatine kinase, the bound nitrophenol can be utilized to monitor a putative conformational change such as that resulting from the inactivation with MMTS. Moreover, the bound nitrophenol spectral change caused by the inactivation by iodoacetamide is different from that shown in Figure 1 for the reaction of MMTS (Laue and Quiocho, unpublished data). It is highly possible that the inactivation of creatine kinase by thiol-specific reagents is partly due to the extent of the accompanying structural rearrangement in addition to the steric presence of a reactant. We have unpublished data which show that the spectrum of the nitrophenol-labeled enzyme that has been largely inactivated with 2 equiv of MMTS can still be perturbed by substrates, but these perturbations were, as expected, different from those shown in Figure 6 for the studies with the active MNP-labeled enzyme.

The unusual selectivity and reactivity of 2 equiv of chromophore toward creatine kinase cannot be fully accounted for or assessed presently. Recently, methylmercury chloride has been shown to react with a pair of thiol groups of creatine kinase without the loss of activity (Smith et al., 1975). Furthermore, yeast 3-phosphoglycerate kinase (Stinson, 1974) and *lac* repressor protein (Yang and Matthews, 1976; Friedman et al., 1976) can be reacted with 2-chloromercuri-4-nitrophenol without the loss of activity and the chromophore can be used to detect ligand-induced conformational changes. The apparent specificity of organomercurials is being exploited to introduce other probes to creatine kinase which might allow further study of substrate binding and possibly the determination of the approximate location of the MNP-specific thiol relative to substrate binding site.

Acknowledgment

We are grateful to Judy Rockel for some technical assistance and to Dr. K. S. Matthews for use of instruments.

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