Affinity Labeling of Creatine Kinase by N-(2,3-Epoxypropyl)-N-amidinoglycine*

Michael A. Marletta† and George L. Kenyon§
From the Departments of Pharmaceutical Chemistry and Biochemistry and Biophysics and the Cardiovascular Research Institute, University of California, San Francisco, California 94143

The compound N-(2,3-epoxypropyl)-N-amidinoglycine (epoxycreatine) was synthesized to probe the active site of rabbit muscle creatine kinase (EC 2.7.3.2) because of its structural similarity to the normal substrate creatine and because of the reactivity of its epoxide moiety toward nucleophiles. In the presence of epoxycreatine and in the absence of other substrates, complete inhibition of enzymatic activity is observed which does not return after exhaustive dialysis. The inhibition follows saturation kinetics. At 0°C (pH 7.45), K_{inact} is 335 mM compared to a K_{i} value of 24 mM for creatine. The inactivation time at varying epoxycreatine concentration is 4.2 min. In a competitive manner, creatine protects the enzyme from inactivation by epoxycreatine. Product studies indicate that epoxycreatine is also a substrate for the enzymatic reaction in the presence of both MgATP and ATP. The partitioning of epoxycreatine between phosphorylation by MgATP and inactivation of the enzyme was followed spectrophotometrically at 25°C and found to be 15 turnovers/inactivating event. The inactivation half-time in the presence of MgATP is slightly greater than that when MgADP is present, which also is slightly greater than that of epoxycreatine alone. The so-called transition state analogue complex of MgADP=NO_{3} + creatine provides strong protection against inactivation by epoxycreatine. The stoichiometry of the reaction, determined with ^{14}C-labeled epoxycreatine, is one site modified per subunit of enzyme, even in the presence of excess reagent. These results indicate that epoxycreatine is binding at the active site and that it is therefore an affinity label.

Creatine kinase (adenosine 5'-triphosphate:creatine N-phosphotransferase, EC 2.7.3.2) catalyzes the reversible transfer of a phosphoryl group from ATP to creatine. The enzyme, both soluble and membrane-bound, is found in abundant supply in both muscle and brain tissues and is generally considered to be important in maintaining constant ATP levels in such tissues (2, 3). Creatine kinase consists of two apparently identical subunits with the molecular weight of the dimer being 82,000. It requires a divalent metal ion for catalysis. In this paper, we have examined the rabbit skeletal muscle enzyme exclusively.

Chemical modifications of creatine kinase from rabbit skeletal muscle have focused mainly on the reactive sulfhydryl group, and a variety of reagents are known to block that functionality (4-13). Other studies have involved modification of a lysine residue (9, 14-16), an arginine residue (17, 18), and a histidine residue (19). Creatine kinase must rank as one of the most thoroughly studied enzymes with respect to both reversible and irreversible inhibition (20). Until the present work (1), however, none of the irreversible inhibitors has been an active-site-directed substrate analogue (affinity label).

Our efforts to synthesize an affinity label structurally related to creatine were stimulated by the lack of information concerning the active site of creatine kinase. The complete primary amino acid sequence is not yet known; in fact, the only such structural information that has been reported is a sequence containing 26 amino acids around the reactive sulfhydryl group (21, 22). An affinity label, owing to its specific interaction with the enzyme, should begin to fill a large gap in our knowledge of the active site of this enzyme that is so important in the bioenergetics of muscle action. Moreover, the ability to inactivate creatine kinase with high selectivity in vivo should permit detailed investigations concerning the bioenergetics of ATP utilization in muscle action without the complicating features of the ATP = phosphocreatine interconversion (23).

The design of the creatine-based affinity label, epoxycreatine, resulted from detailed investigations of the bulk tolerances of the active site of creatine kinase. Preliminary studies had shown that both glycocyamine (24) and N-ethylglycocyamine (25) could act as substrates. 

![Diagram](http://www.jbc.org/)

where R = H, glycocyamine, CH_{3}, creatine, CH_{2}CH_{3}, N-ethylglycocyamine; CH_{2}CH_{2}CH_{3}, N-propylglycocyamine; CH_{2}CH=CH_{2}, epoxycreatine.

More detailed and quantitative studies revealed the extent

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to which the active site could tolerate derivatives of creatine where the N-methyl group had been altered (26). Thus, it was found, for example, that the N-ethyl and N-propyl homologs of creatine were substrates in the enzymatic reaction with $V_{max}$ values relative to creatine of 32 and 1%, respectively (27).

The epoxide moiety was chosen as the reactive component of the affinity label because of its reasonable stability in neutral aqueous solutions and its anticipated substantial reactivity with nucleophiles when bound to the active site of the enzyme. The structure of epoxycreatine is, of course, sterically rather similar to that of the N-propyl analogue of creatine. Therefore, it was anticipated that epoxycreatine would also be able to bind at the active site and then possibly inactivate the enzyme.

**MATERIALS AND METHODS**

Allylamine and 3-amino-1,2-propanediol were both purchased from Aldrich; iodoacetic acid was from Matheson, Coleman and Bell; [14C]iodoacetic acid (1.5 mCi, 13.9 mCi/mmol) was from New England Nuclear; creatine monohydrate was from Eastman; cyanamide and magnesium acetate were both from J. T. Baker; sodium tungstate dihydrate was from Afa; bovine serum albumin, adenosine 5'-triphosphate, lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, and NADH were all from Sigma; Hepes was from Calbiochem; all solvents and 58% aqueous ammonia were from Mallinckrodt. The 50% hydrogen peroxide solution was a generous gift from Du Pont. Water used for all buffer solutions was distilled and deionized, and water used as the solvent for high performance liquid chromatography was glass-distilled. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California Berkeley.

Creatine kinase was isolated from fresh rabbit skeletal muscle as described by Kuby et al. (2) in their Procedure E. The enzyme obtained from this purification had a specific activity of 135 mg protein/min as assayed by the pH-stat method. The spectrophotometric coupled enzyme assay of McLaughlin et al. (27) was also utilized for the studies indicated. The concentration of creatine kinase was determined spectrophotometrically at 280 nm using the extinction coefficient of 7.1 x 10³ M⁻¹ cm⁻¹ as reported by Mahowald and coworkers (4). Both radioactivity scintillation counting procedures and NMR spectrometers used have been described previously (12).

**Synthesis of N-(2-Propenyl)glycine**

A solution of iodoacetic acid (14.9 g, 0.08 mol) in 40 ml of water was added dropwise to ice cold, rapidly stirred allylamine (38.6 g, 0.68 mol). The reaction mixture was allowed to stand for 20 h after which the water and excess allylamine were both removed in vacuo leaving a viscous, amber-colored oil. The oil was dissolved in 40 ml of absolute ethanol and added slowly to a stirred solution of 50 ml of absolute ethanol and 470 ml of acetone. The solution was allowed to stand at 4°C for 12 h, during which time a white precipitate formed. The white powder (4.8 g) was filtered, dried giving a 46% yield in the reaction, and based on that, 95% of the theoretical amount of epoxycreatine was obtained after lyophilization. 

**Synthesis of [14C]Epoxycreatine**

A portion of this compound was dissolved in distilled water and placed in a boiling water bath for 5 min. An aliquot was then injected into the HPLC and the retention time was now coincident with that of N-amidino-N-(2,3-dihydroxypropyl)glycine (the diol analogue, see below).

**Hydroxylations**

The synthesis of the radioactively labeled epoxycreatine was exactly the same as that for the cold compound with the label being introduced in the first step of the procedure as [14C]iodoacetic acid (labeled at the carboxylate carbon).

N-(2,3-Dihydroxypropyl)glycine—This was prepared from a solution of iodoacetic acid (11.18 g, 0.06 mol) in 35 ml of water which was then added dropwise to an ice cold solution of 3-amino-1,2-propanediol (42.0 g, 0.47 mol) in 20 ml of water. The reaction mixture was allowed to stand for 30 h after which time the water and excess 3-amino-1,2-propanediol were both removed in vacuo leaving a viscous, pale yellow oil. The oil, now dark brown in color, was heated on a steam bath in 20 ml of absolute ethanol and added slowly to a warm solution of 50 ml of absolute ethanol and 100 ml of acetone. This solution was allowed to stand at 4°C for 24 h, during which time a brown precipitate formed. The brown powder was filtered, dried, and recrystallized from 95% ethanol. The white crystals which formed were filtered and dried giving 2.5 g of the product (28% yield), m.p. 152-153°C (decomposition).

**N-Amidino-N-(2,3-dihydroxypropyl)glycine**—This was prepared by dissolving fresh ether-extracted cyanamide (1.05 g, 0.025 mol) in a solution of 2 ml of water and 4 drops of an aqueous NH₄OH solution (28%). N-(2,3-Dihydroxypropyl)glycine (3.3 g, 0.020 mol) was then added in small aliquots and swirled after each addition until a white precipitate formed. The white crystals which formed were filtered, washed with cold absolute ethanol and dried giving 4.13 g of product (80% yield), m.p. 244-245°C (decomposition).

**N-Amidino-N-(2,3-epoxypropyl)glycine (Epoxycreatine)**

N-Amidino-N-(2-propyl)glycine (1.5 g, 9.5 mmol) was dissolved in 10 ml of 50% H₂O₂. To initiate the reaction, sodium tungstate dihydrate (0.13 g, 0.44 mmol) was added and the reaction solution was stirred at room temperature for 90 min. When the sodium tungstate was added, the colorless solution immediately turned pale yellow and remained so during the course of the reaction. After the course of the reaction, after 90 min, 100 ml of acetone was added and the white powdery precipitate (1.5 g) was filtered, washed with acetone, and dried. The product was purified by high performance liquid chromatography using a reverse phase Waters microcrystalline Bondapak C-18 preparative column (0.7 x 30 cm) with water as the eluent. The crude product was dissolved in 20-mg lots. Each 20-mg sample was dissolved in 150 ml of water just prior to injection. The flow rate was 2.0 ml/min and a refractive index detector was used. The product solution had a retention time of 8.5 min and the starting material eluted at 11 min. The product-containing solution once collected was kept on ice until all injections were made, and then it was frozen, lyophilized, and stored at room temperature under vacuum in a dessicator. The refractive index detector indicated a 46% yield in the reaction, and, based on that, 80% of the theoretical amount of epoxycreatine was obtained after lyophilization.
clear solution was obtained. When the addition of N-(3,3-dihydroxypropyl)glycine was complete, the flask was sealed with a greased ground-glass stopper and swirled again until the solution was clear. Crystals began to form after 20 h. After 5 days, the crystals and residual solvent were ground in a mortar, filtered, on a sintered glass funnel until dry, washed on the funnel with 50 ml of acetone, and finally dried again. The white powder (2.5 g) was recrystallized by suspending it in boiling 95% ethanol and by dropwise addition of water until the solution became clear. The white crystals were filtered, washed with cold absolute ethanol, and dried, giving 1.43 g (37% yield), m.p. 185-187°C (decomposition).

Yield), m.p. 185-187°C (decomposition). Washed with cold absolute ethanol, and dried, giving 1.43 g (37% yield), m.p. 185-187°C (decomposition).

Next, the enzyme was added to initiate the reaction, and it was allowed to proceed for a total of 60 min. The reaction was quenched by the addition of a solution of 2,4-dinitrofluorobenzene (50 μl, 0.1 M) in 2-propanol (28). Aliquots (5 μl) of the epoxycreatine and creatine reactions, removed at various times, were each spotted on the polyethyleneimine-cellulose plates, developed in a 1.2 M NaCl solution, and the spots were visualized.

**Stoichiometry of Creatine Kinase-Epoxycreatine Reaction**

To a vial containing 14C-labeled epoxycreatine (1.73 mg, 100 mM final concentration) was added 100 μl of a solution of creatine kinase (17.1 mg/ml, 209 μM) in 0.01 M Hepes buffer, pH 7.45, at 0°C. The reaction was carried out at 0°C and the reaction mixture consisted of the following; 50 μl of Na2ATP (pH 8.5), 50 μl of 0.4 M MgAc2, 0.35 ml of 0.1 M glycine buffer (pH 9.0), 50 μl of creatine kinase (26.1 mg/ml), and 5 mg of epoxycreatine. All components of the reaction mixture were combined except for the enzyme. A control reaction was set up which consisted of the same components except that creatine (5 mg) replaced the epoxycreatine. The enzyme was added to initiate the reaction, and it was allowed to proceed for a total of 60 min. The reaction was quenched by the addition of a solution of 2,4-dinitrofluorobenzene (50 μl, 0.1 M) in 2-propanol (28). Aliquots (5 μl) of the epoxycreatine and creatine reactions, removed at various times, were each spotted on the polyethyleneimine-cellulose plates, developed in a 1.2 M NaCl solution, and the spots were visualized.

**Per Cent Residual Activity versus Epoxycreatine Binding**

To 1.0 ml of a creatine kinase solution (17.1 mg/ml, 209 μM) at 0°C in 0.1 M Hepes, pH 7.4, was added at time 0 μC-labeled epoxycreatine (5.7 mg, 38.6 mM). Aliquots (100 μl) were withdrawn at specified times and immediately passed through a column (7 mm × 17 cm) containing Sephadex G-25 medium grade gel that had been equilibrated with 0.01 M Hepes, pH 7.4, and creatine (80 mM). The eluant was monitored by observing the absorption at 280 nm. The protein-containing eluant was collected, diazylized against three changes of 0.01 M Hepes (1.0 liter each), and assayed for creatine kinase specific activity, and then the amount of radioactivity bound was determined by scintillation counting. The entire procedure following the initial incubation was carried out at 4°C.

**Removal of the Label from Creatine Kinase**

**NaOH Treatment**—to a sample of the blocked protein (0.6 mg/ml, 200 μl) was added 0.2 N NaOH (200 ml). The solution was mixed immediately and then left to stand for 12 h at room temperature. After that time, the solution was diazylized against 1.0 1 of deionized, distilled water (three changes for 5 h each). The protein concentration was determined spectrophotometrically, and then the sample was counted.

**Comparison of the Rate of Removal of Label by NaOH and NH2OH**—the same protein sample was used in this experiment as was used above. To one protein sample (0.6 mg/ml, 400 μl) was added 2 M NH2OH, pH 9.0 (400 μl), and to the other protein sample (0.6 mg/ ml, 400 μl) was added 0.10 M glycine solution, pH 9.0 (400 μl). The samples stood for 4 h after which time they were diazylized against 1.0 liter of deionized, distilled water (three changes for 5 h each). Protein concentration was measured spectrophotometrically and the samples were counted.

**Effect of Other Substrates on the Inactivation Rate**

A sample of creatine kinase in 0.01 M Hepes, pH 7.45, (2.0 mg/ml, 50 μl) was added to a solution of epoxycreatine (48 mM) and the other substrate or substrates also in 0.01 M Hepes, pH 7.45. As in the typical inactivation experiments, aliquots (5 μl) were withdrawn at noted times and assayed on the pH-stat.

**Partitioning of the Turnover versus Inactivation Reactions**

The following solutions in 0.01 M Hepes at pH 7.5 were added to a cuvette: magnesium acetate (40 mM, 0.15 ml), ATP (40 mM, 20 μl), P-enolpyruvate (10 mg/ml, 20 μl), NADH, (10 mg/ml, 10 μl), pyruvate kinase (10 mg/ml, 2.5 μl), lactate dehydrogenase (10 mg/ml, 25 μl), and epoxycreatine (10 mg/0.8 ml of buffer). Various volumes of a creatine kinase solution (0.68 mg/ml) were added to initiate the reaction, which was carried out at 25°C with observation of the absorbance at 340 nm.

**RESULTS**

**Synthesis and Characterization of Epoxycreatine**

The synthesis of epoxycreatine presented few difficulties; however, attempts to purify it by standard synthetic methodology were not successful. Purification was finally accomplished by preparative scale, reverse phase HPLC.

The structure proof of epoxycreatine rests on the microanalysis, the proton NMR spectrum with the characteristic upfield absorption of the epoxide ring protons, and the HPLC retention time relative to those of starting vinlylcarnitine and the corresponding diol analogue. The diol analogue of epoxycreatine was synthesized via a different synthetic route, and both its HPLC retention time and NMR spectrum were determined so that it could be used for comparison purposes in the isolation and characterization of epoxycreatine. The epoxide would be predicted to elute from the reverse phase HPLC column between the diol and the olefin, and this was what was found. Upon boiling in water, the epoxide was converted quantitatively to the diol analogue, a conversion which led to the expected changes both in the HPLC retention time and in the proton NMR spectrum. This provided further evidence for the epoxide’s structure.
were performed at 0°C, 0.1 M Hepes (pH 7.45), epoxycreatine concentration of 48 mM, and creatine kinase concentration of 1 mg/ml. Under these conditions, the enzyme was completely inactivated in about 4.25 h. The initial and later studies of the epoxycreatine-creatine kinase interactions were performed at 0°C because it was found that epoxycreatine was relatively stable to hydrolysis at that temperature for at least 12 h and that therefore the competing hydrolysis did not interfere with the quantitation of the rate of inactivation. The results of these experiments at five different epoxycreatine concentrations are shown in Fig. 1.

The kinetic arguments which support the reaction of an inhibitor at the active site of an enzyme were developed independently by Kitz and Wilson (29), Main (30), and Fahrney and Gold (31). The kinetic derivations, along with other criteria which must be met if an inhibitor is active site-directed, have been summarized by Meloche (32).

The affinity label, if it is indeed reacting at the active site, should in the initial binding step act similarly to the normal substrate; that is, there should be a dissociable complex formed between the affinity label and the enzyme. This complex formation is represented by the following equation:

\[ E + I \overset{h_1}{\underset{h_{-1}}{\rightleftharpoons}} EI \overset{k_2}{\rightarrow} E_{\text{inact}} \]  

where \( E \) represents free enzyme, \( I \) is the inhibitor (in this case, epoxycreatine), \( EI \) is the enzyme-inhibitor complex, and \( E_{\text{inact}} \) is the inactivated enzyme. Using the steady state assumption, Meloche (32) has derived the following rate expression for inactivation:

\[ t_{1/2} = \frac{1}{I} \left( T_{1/2} K_{\text{inact}} \right) + T_{1/2} \]  

where \( t_{1/2} \) is the inactivation half-time at a given inhibitor concentration \([I]\). \( T_{1/2} \) is the minimum inactivation half-time at infinite inhibitor concentration, and \( K_{\text{inact}} \) is \((k_+ + k_2)/k_1\). \( K_{\text{inact}} \) is also the concentration of the inhibitor that gives the half-maximal inactivation rate and presumably half-saturates the enzyme (32). If the interaction of the inhibitor and enzyme first forms a dissociable complex, then a plot of \( t_{1/2} \) versus \( 1/[I] \) should give a straight line with an intercept \( T_{1/2} \).

Values of \( t_{1/2} \) were obtained graphically at various concentrations of epoxycreatine as shown in Fig. 1. A plot of \( t_{1/2} \) versus \( 1/[I] \) according to Equation 2 is shown in Fig. 2.

Based on the observation that the double reciprocal plot is linear and intersects the ordinate at a point greater than zero, epoxycreatine apparently does form a dissociable complex prior to inactivation of the enzyme as described by Equation 1. The minimum inactivation half-time (\( T_{1/2} \)) is 4.2 min. This value corresponds to a pseudo-first order rate constant of

\[ k = \frac{\ln 2}{4.2\ \text{min}} = 2.8 \times 10^{-1} \text{s}^{-1} \]

The value of \( K_{\text{inact}} \) can be obtained either from the slope of the line or from the negative of the reciprocal of the intercept on the abscissa. Therefore, \( K_{\text{inact}} = 355 \) mM.

**Substrate Protection of the Inhibition**

If indeed epoxycreatine is inactivating creatine kinase by an irreversible reaction at the creatine binding site, then creatine present in solution should protect competitively against the inhibition. Equations describing this can be derived in a similar fashion as before using the steady state approximation (32). The reaction scheme is shown below:

\[ E + I \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} EI \overset{k_2}{\rightarrow} E_{\text{inact}} \]

\[ S \overset{k_3}{\rightarrow} ES \]

where \( S \) represents the normal substrate (creatine) and \( ES \) is the enzyme-substrate complex. The steady state approximation affords the linear expression:

\[ t_{1/2} = \frac{1}{I} T_{1/2} \left( K_{\text{inact}} + \frac{K_{\text{inact}} [S]}{K_s} \right) + T_{1/2} \]  

where \( K_s = k_{-3}/k_3 \) and represents the apparent dissociation constant of the enzyme-substrate complex. This equation, when plotted as the \( t_{1/2} \) versus \( 1/[I] \) at constant levels of \([S]\), should result in a series of straight lines whose slopes are determined by the substrate concentration, and the lines should intercept the ordinate at the minimum inactivation half-time. In addition, the \( K_s \) value for the substrate can be determined from the slopes of the lines. The results are summarized in Table I.

**Fig. 1.** Loss of creatine kinase activity upon incubation with epoxycreatine. Conditions were as follows: creatine kinase (1.0 mg/ml) in 10 mM Hepes (pH 7.5) at 0°C. The epoxycreatine concentrations are noted on the figure.

**Fig. 2.** The half-time of inactivation of creatine kinase as a function of the reciprocal of the epoxycreatine concentration. The half-times of inactivation were obtained from Fig. 1.
Stoichiometry of Epoxycreatine-Creatine Kinase Interaction

The stoichiometry of binding of epoxycreatine to creatine kinase was determined by the use of 14C-labeled epoxycreatine. The specific activity of epoxycreatine used was 2.5 × 10⁻¹ mCi/mmol. The protein concentration was determined spectrophotometrically as stated under "Materials and Methods" and was compared to the Lowry method of protein determination (34). In preliminary experiments, both methods yielded the same results and, thereafter, the spectrophotometric determination was used because of its greater simplicity.

The particular experiments were carried out at an epoxycreatine concentration of 100 mM at 0°C for 20 h. The half-time at this concentration was 32 min and, therefore, the reactions were carried out for 37.5 half-times. The results found of 1.96 and 2.02 mmol of 14C label/mmol of creatine kinase lead to an average value of 1.99.

Enzyme Activity versus Epoxycreatine Bound

In an experiment to check if the subunits of creatine kinase react at the same rate with epoxycreatine and if modification of both subunits is necessary to obliterate activity, the enzyme was treated with the inhibitor, assayed for enzymatic activity, and counted to determine the amount of epoxycreatine bound. The results are shown in Fig. 3. It should be noted that five points were taken at times after 2.0 mmol of label had been bound/mmol enzyme, all of which indicated no further reaction, and the points were weighted accordingly. The results clearly indicate that both subunits react with epoxycreatine at the same observable rate and that incorporation of 2 mmol of label/mmol of enzyme (i.e. 1 mmol/mmol of subunit) is necessary to obliterate enzymatic activity.

Effect of Other Substrates on the Inactivation Rate

The concentration of epoxycreatine used in these experiments was 48 mM, and the inactivation half-time was 21 min. The earlier recorded half-time at this concentration was 32 min. The difference was reproducible and has been ascribed to a difference in specific activity of this enzyme preparation, which was prepared at a later date. The results of this study are shown in Table II.

Milner-White and Watts (35) first postulated that the abortive ternary complex of creatine-MgADP-NaNO₃, when bound to creatine kinase, forms an extraordinarily tight complex, presumably because the planar NO₃⁻ mimics the transferred phosphoryl group, and this ternary complex may therefore be considered as a transition state analogue complex (36). A number of research groups have investigated this idea since

### Table I

<table>
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<tr>
<th>[Creatine]</th>
<th>Slope</th>
<th>Intercept</th>
<th>K_0</th>
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<td>min⁻¹</td>
<td>mM</td>
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<td>4.2 ± 1.7</td>
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<tr>
<td>20</td>
<td>1568</td>
<td>3.3 ± 0.7</td>
<td>46</td>
</tr>
<tr>
<td>40</td>
<td>1804</td>
<td>1.8 ± 0.8</td>
<td>20</td>
</tr>
</tbody>
</table>

* Determined from plots of t¹/² versus [epoxycreatine]⁻¹ in the presence of two different concentrations of creatine. Data was analyzed with a least squares fit program with a weighting subroutine (33).

### Table II

<table>
<thead>
<tr>
<th>Epoxycreatine</th>
<th>Creatine</th>
<th>MgATP</th>
<th>MgADP</th>
<th>NO₃⁻</th>
<th>t₁/²</th>
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<tbody>
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<td>mM</td>
<td>mM</td>
<td>mM</td>
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<td>4</td>
<td>8</td>
<td>8</td>
<td>366</td>
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</table>

Effect of Oxycreatine as a Substrate

The limited quantities of epoxycreatine that were available at the time precluded the use of the pH stat assay, and so the method of Rowley and Kenyon (28) was used. The results indicated that epoxycreatine had been phosphorylated and that there was an increase in the amount of ADP generated at the expense of ATP, based on comparison with a control. For epoxycreatine to be a substrate in the enzymatic reaction it must bind at the active site and, therefore, this experiment clearly shows that epoxycreatine is capable of interacting at the active site of the enzyme.

### Fig. 3

Loss of creatine kinase activity upon incorporation of 14C-epoxycreatine. Conditions were as follows: creatine kinase (17 mg/ml, 209 μM) and epoxycreatine (39 mM) in 10 mM Hepes (pH 7.5) at 0°C. For other details see "Materials and Methods."

### Fig. 4

Protection from inactivation by the MgADP-NaNO₃-creatine complex. Conditions were as follows: A, creatine kinase (1.0 mg/ml), epoxycreatine (48 mM), ADP (8 mM), MgAc₂ (8 mM), NaNO₃ (8 mM), and creatine (40 mM) in 10 mM Hepes (pH 7.5) at 0°C; B, same, except ADP, MgAc₂, NaNO₃, and creatine were all deleted.
then and have generally found supporting evidence. As is
evident from the data of Table II and Fig. 4, this complex
apparently protects the enzyme from inactivation by epoxycrea-
tine to a very large degree.

Removal of the Affinity Label from Creatine Kinase

Treatment with NaOH—Creatine kinase that had been
treated with [14C]epoxycreatine for another experiment was
utilized, and it contained 1.5 mmol of 14C label/mmol of
enzyme. The protein was treated for 12 h at 25°C with 0.1 N
NaOH. The protein sample was counted to determine the
amount of radioactivity present, and the ratio was found to be
0.2 mmol of 14C label/mmol of enzyme; therefore, 87% of the
radioactive label was lost. The ease and mild conditions which
causc the label's removal suggest that perhaps a carboxylate
group of creatine kinase had been modified.

Comparison of the Effect of NaOH and NH2OH—Two
samples of [14C]epoxycreatine-treated creatine kinase were
treated analogously except one was in glycin buffer, pH 9.0,
and the other in 2.0 M NH4OH, pH 9.0. The samples were
treated for 5 h and then counted. The results showed that the
base-treated sample lost 18% of the label while the NH4OH-
treated sample lost 70% of the label. The results clearly
indicate that NH4OH accelerates the rate of removal of the
label and further implicate a carboxylate residue of the en-
zyme as being the nucleophile towards epoxycreatine.

Partitioning of the Turnover versus Inactivation Reactions

The results of this experiment are depicted in Fig. 5, which
shows the decrease in the absorbing at 340 nm with time. At
25°C the enzyme was inactivated in 19.5 min, and the ratio of
turnover versus inactivation was 15.

DISCUSSION

The minimal criteria to which an active site-directed irre-
versible enzyme inhibitor must conform are: 1) the inactiva-
tion must be complete; 2) activity must not return upon
exhaustive dialysis; 3) the inactivation should obey saturation
kinetics; 4) the normal substrate of the enzymatic reaction
should protect against the inactivation in a competitive man-
ner; and 5) the irreversible binding of the inhibitor should be
stoichiometric, i.e. 1 inhibitor molecule bound/active site. As
the results indicate, epoxycreatine conforms to all of these
criteria. An additional demonstration that epoxycreatine is
capable of interaction with the active site of creatine kinase is
the fact that epoxycreatine can serve as a substrate in the
enzymatic reaction. This is not surprising as the design of the
molecule was based on substrate analogue studies which had
shown where on the creatine structure increased bulk could
be tolerated by the enzyme. Evidence for this phosphorylation
of epoxycreatine came from the observation that ADP was
being formed at the expense of ATP concomitantly with
formation of the putative phosphorylated epoxycreatine. At
the same time, no inorganic phosphate (based on the control)
was being generated.

The value for  \( K_{\text{inact}} \) was determined to be 335 mM. This
value seems reasonable when compared to other relevant
experimental data. Maggio and Kenyon1 have found the  \( K_\text{S} \)
of the N-propyl analogue of creatine to be 395 mM at 30°C with
the beef skeletal muscle isoenzyme. While the  \( K_\text{S} \) values vary
somewhat from isoenzyme to isoenzyme, the  \( K_{\text{inact}} \) seems to
be in general agreement with this  \( K_\text{S} \) value. Also, the  \( K_\text{S} \)
value was determined at 30°C and the  \( K_{\text{inact}} \) value with epoxycrea-
tine at 0°C. The  \( K_\text{S} \) of creatine itself is 24 ± 5 mM at 30°C
(37); however, the value at 0°C has not been determined.

The first order rate constant for the inactivation is 2.8 ×
10^{-3} s^{-1} which is equivalent to 9.9 h^{-1}. This value can be
compared to published data for the aqueous reactions of
epoxides (38). The value of 9.9 h^{-1} compares very favorably
with values for the acid-catalyzed addition of water to a
variety of simple epoxides, which show rate constants in the
5- to 50-h^{-1} range. The literature data was determined at 37°C
and that for epoxycreatine at 0°C. Considering the neutral pH
and lower temperature used in the epoxycreatine inactivation
of creatine kinase relative to these model studies, the enzy-
matic inactivation is a remarkably rapid process, which is
characteristic of unimolecular rate processes involved in affin-
ity labeling. The enzyme itself could hydrogen bond to the
oxygen of the epoxide ring and therefore mimic, to some
extent, the acid-catalyzed reaction. This is illustrated below:

\[
\begin{align*}
&\text{H}_N^+ \text{NH}_2 \quad \text{H} \quad \text{O} \\
\text{O}_\text{C} &\text{H} \quad \text{H} \\
\text{H}^+ &\text{NH}_2 \quad \text{H} \quad \text{O} \\
\text{O}_\text{C} &\text{H} \quad \text{H} \\
\end{align*}
\]

where the group Y hydrogen bonds to the oxygen and the
group X reacts with the epoxide ring carbon. The attack of X
is shown at the least sterically hindered position on the ring;
however, considering the potential constraints at the active
site, reaction at the other position is certainly possible.

As indicated earlier in Equation 3, it is possible to determine
the dissociation constant (  \( K_\text{D} \) ) for creatine from the substrate
protection experiments. The  \( K_\text{D} \) values at 0°C (see Table I)
for 20 and 40 mM creatine are 46 and 20 mM, respectively. It
is important to note the proximity of these values to the
known  \( K_\text{D} \) value of 24 ± 5 mM for creatine (37) determined by
steady state kinetic measurements in the absence of inhibitor

1 E. T. Maggio and G. L. Kenyon, unpublished results.
at 30°C. From the scatter of the data, the errors in the $K_i$ values determined in the presence of epoxycreatine are considerable, and the differences among these values may in reality not be great. It would have been desirable to have used higher concentrations of creatine in these substrate protection experiments, however, the low solubilities of both creatine and epoxycreatine precluded such studies.

The partitioning experiment points out the relative efficiencies of epoxycreatine to turn over in the phosphorylation reaction and to inactivate the enzyme. The result of this experiment indicates that epoxycreatine is fairly efficient in its reaction with the enzyme with the inactivating event occurring approximately 7% of the time. It should be noted that once the full complement of substrates is available, the enzyme will turn over and produce the putative product, phosphoepoxycreatine. The interaction of this latter product relative to epoxycreatine with the enzyme is not yet known.

From the results shown in Fig. 3, it is clear that epoxycreatine reacts at each subunit of the dimer at an equal observable rate. These results imply that the binding of epoxycreatine at one subunit does not affect binding at the other. This is in line with results of Hammes and Hurst (39) who have reported that creatine binding causes very little change in conformation one subunit does not affect binding at the other. This is in that once the full complement of substrates is available, the enzyme will turn over and produce the putative product, phosphoepoxycreatine. The interaction of this latter product relative to epoxycreatine with the enzyme is not yet known.

In Table II, the results for the effect of other substrates on the inactivation rate show that the half-times increase in the presence of the nucleotide substrates in the following fashion: control $< \text{MgADP} < \text{MgATP}$. One might have expected the half-times to have decreased in the presence of the nucleotides because of the synergistic effect of the nucleotides on creatine (and, presumably, epoxycreatine) binding (37). However, based on the efforts of Kenyon and Struve (40), Phillips and Quiocho (unpublished results cited in Ref. 41) and Struve et al. (42), it has been suggested by use of a highly reactive creatine analogue (42) that the nitrogen trans to the carboxymethyl group (cis to the methyl group) of the creatine analogue is the one that is phosphorylated in the enzymatic process. The nitrogen that is presumably phosphorylated is thus on the same side of the molecule as the epoxypropyl group of epoxycreatine. A possible interpretation of these results, then, is that the epoxypropyl group overlaps partially in the nucleotide binding site and that therefore the increase in inactivation half-time in the presence of the nucleotides is a steric phenomenon.

The result of protection with the so-called transition state analogue complex of MgADP$^-$, NO$_2^-$, creatine ($t_{1/2} = 366$ min) shows that in this tight complex epoxycreatine is presumably prevented from ready access to the active site and that, therefore, the rate of inactivation is very slow. This result provides strong evidence that the inactivation process is taking place at the active site.

In earlier studies with other enzymes it has been found for several epoxide-containing affinity labels that a carboxylate group of either an aspartic or glutamic acid residue was modified on the given target enzyme (43-45). These reactions with the carboxylate groups created ester functionalities on the enzymes’ surfaces. In these cases, the investigators usually treated the inactivated enzyme with dilute alkaline solutions and studied the effect of such base treatment on the enzyme-affinity label adduct. In cases when the label was labile in alkaline solutions, the next step usually was to investigate the effect on this label’s removal rate by added hydroxylamine. If an ester hydrolysis had occurred, then addition of hydroxylamine was expected to increase this rate of removal.

The evidence so far for epoxycreatine, although not definitive, also suggests that a carboxylate group of either an aspartic or glutamic acid residue has been modified. The label is removed rather easily in an aqueous alkaline solution and the rate of that removal proceeds much faster in an aqueous hydroxylamine solution. Efforts are currently underway to determine the amino acid residue or residues labeled by epoxycreatine.

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