Iodine Oxidation of the Sulfhydryl Groups of Creatine Kinase*

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ABSTRACT: Evidence is presented for oxidation of the cysteinyl residues of creatine kinase to the level of sulfenate, for the reversal of this oxidation with mercaptans, and for the correlation of these changes with alterations in the specific binding and catalytic properties of the enzyme. In the presence of excess iodine, a total of 12 equiv of iodine/mole of creatine kinase was consumed in the oxidation of six sulfhydryl groups of this protein.

The linear relationship found to exist between the loss of sulfhydryl groups and the consumption of iodine indicates that these six sulfhydryl groups were oxidized to the same extent, and that the reaction was specific for the oxidation of cysteinyl side chains. When [^{125}I]I_2 was used for the oxidation, the oxidized protein after gel filtration contained no significant amount of ^{125}I. Thus, although the oxidation may proceed through a sulfenyl iodide intermediate, there is no indication of the formation of a stable protein sulfenyl iodide. Evidence obtained thus far is consistent with representation of the oxidation product as a sulfenic acid formed by hydrolysis of a sulfenyl iodide. The addition of 4 equiv of iodine/mole of enzyme leads to the oxidation of only two sulfhydryl groups, but results in the loss of all catalytic activity. The iodine-inactivated enzyme shows no changes in ultraviolet spectrum, and binds the substrate magnesium adenosine diphosphate, although not so strongly as the native protein. The inactive enzyme, prepared by the reaction with slightly less than 4 equiv of iodine/mole, can be reactivated by incubation with mercaptoethanol or dithiothreitol. This reactivation presumably occurs through the intermediate formation of a mixed disulfide with the subsequent regeneration of the original sulfhydryl group. It is apparent from the stoichiometry, the absence of iodine in the oxidized protein, and the reactivation with mercaptans, that the product of the oxidation of the sulfhydryl groups is a sulfenyl function, presumably the sulfenic acid group. This alteration of the SH groups in the active site produces little or no change in the physical properties measured, and yet is sufficient to abolish all enzymatic activity.

The use of iodine as a protein oxidant is complicated by the variety of iodine reactions with proteins. In addition to cysteinyl residues, tyrosinyl, histidinyl, and tryptophanyl residues will react with solutions of iodine under appropriate conditions. However, it has been clearly shown that at low temperature and pH, oxidation is limited primarily to cysteinyl residues, particularly if the protein is not exposed to large excesses of iodine over long periods (Cunningham and Nuenke, 1959).

The oxidation of cysteinyl residues is generally regarded as leading to disulfide-bond formation. However, studies of human serum albumin (Hughes and Straessle, 1950), of 3-phosphoglyceraldehyde dehydrogenase (Rafter, 1957), of TMV (Fraenkel-Conrat, 1950), and of lactoglobulin and ovalbumin (Cunningham and Nuenke, 1959) suggest oxidation of cysteinyl residues to products other than disulfide. In the latter three proteins the product of the oxidation was shown to be sulfenyl iodide. The chemical nature of the lactoglobulin sulfenyl iodide has received especially careful study (Cunningham and Nuenke, 1960, 1961; Cunningham, 1964).

The iodine oxidation of cysteinyl residues in proteins can be compared with the reaction sequence proposed for the oxidation of free cysteine with the stepwise formation of cystine, cysteinesulfenic acid, cysteinesulfinic acid, and cysteinesulfonic acids, although the exact mechanisms may not be identical (Danehy, 1967).

$$2RSH + I_2 \longrightarrow RSSR + 2I^- + 2H^+$$

$$RSSR + 2H_2O + I_2 \longrightarrow 2RSOH + 2I^- + 2H^+$$

$$RSOH + H_2O + I_2 \longrightarrow RSOH_2 + 2I^- + 2H^+$$

$$RSO_2H + H_2O + I_2 \longrightarrow RSO_3H + 2I^- + 2H^+$$

where R = $CH_2CH(NH_2)COOH$

It has been suggested that protein sulfenyl iodide groups are intermediates in the iodine oxidation of cysteinyl residues to disulfide bonds (Fraenkel-Conrat, 1955). The following sequence of reactions represents the oxidations of cysteinyl side chains through sulfenyl and sulfinyl intermediates (Kharasch, 1961). The oxidation state of the sulfur is indicated in parentheses. Oxidation to the disulfide level in the case of dilute solutions of proteins, however, would require a special prox-

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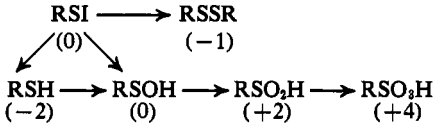

imal relationship between two cysteinyl residues within a single protein molecule.

Creatine kinase has two reactive sulfhydryl groups which have been designated as the "active-site" cysteinyl residues (Mahowald *et al.*, 1962). This is consistent with the understanding that creatine kinase (molecular weight 81,000) consists of two identical monomeric subunits. Other evidence suggests that there may be a total of eight cysteine residues per mole (Yue *et al.*, 1967; Bayley and Thomson, 1967) of vastly different reactivities. In addition to the two residues essential for enzymatic activity, there are four which are relatively unreactive toward or otherwise "protected" from many sulfhydryl reagents, such as iodoacetate, except under denaturing conditions. The remaining two residues are extremely inert (Noltmann *et al.*, 1962) and have been reported on the basis of amino acid analysis of denatured, reduced, carboxymethylated enzyme. The possibility that these two inert residues form a disulfide bridge is considered very unlikely (Yue *et al.*, 1967). They were not observed or considered in our experiments. It was of particular interest to see if these differences in chemical reactivities would result in different products with iodine oxidation.

Since this reaction had been used previously with proteins which have no easily measured specific biological activity, its application to a well-characterized enzyme was aimed toward providing information regarding the functional role of the cysteinyl residues in maintaining its catalytic activity and native conformation.

Materials and Methods

Creatine Kinase. Creatine kinase was isolated from rabbit muscle according to procedure B of Kuby *et al.* (1954) and crystallized by the method of Mahowald *et al.* (1962). Twice-crystalline preparations had a specific activity of 55–60 Kuby units/mg when assayed by the phosphate method (Kuby *et al.*, 1954) and 110–120 μequiv/min per mg as measured by the pH-Stat assay (Mahowald *et al.*, 1962). These preparations were homogeneous when examined in the ultracentrifuge. Protein concentrations were determined spectrophotometrically at 280 mμ assuming $E^{1\%}_{1\,cm}$ 8.8 (Noda *et al.*, 1954; Kuby and Noltmann, 1962). Routine assays were performed by the pH titration method (Mahowald *et al.*, 1962).

Iodine Solutions. Solutions of iodine were prepared in 0.2 M KI and the iodine concentration was determined by absorption at 355 mμ (Cunningham and Nuenke, 1959). [^{125}I] Iodine solutions of the desired specific activity were prepared by equilibration of isotopic iodine (Cambridge Nuclear Corp.) with a solution of iodine in aqueous potassium iodide.

Iodine Uptake Reactions. The reaction of iodine with added protein was followed by the decrease in absorbance at 355 mμ at 2–4°. Reaction mixtures of 2 ml contained 0.2 M KI, 6.2–7.0 × 10^{-5} N iodine, and 0.2 M sodium phosphate (pH 6.1). The protein concentration was selected to maximize the change in absorbance occurring over a 20-min period, with most of the readings in the range from 0.1 to 0.5 optical density units.

A cuvet containing the reaction mixture was allowed 5–10 min for temperature equilibration in the spectrophotometer before the reaction was initiated by the addition of enzyme (0.1–0.8 mg) in 50–100 μl of the phosphate buffer. Readings were continued at 1–2-min intervals until there was no further rapid change in optical density over a 2–3-min period. The disappearance of iodine continued at a slow but finite rate, and the equivalence point was taken as the point to which this rate extrapolated at zero time.

Iodine Titration Reactions. According to the procedure of Cunningham and Nuenke (1959), sequential additions of iodine were made to a cuvet containing 50 mg of creatine phosphokinase, 0.35 M sodium phosphate–0.2 M KI (pH 6.3) in a volume of 2 ml. Small amounts (10–50 μl) of a 0.01884 N solution of iodine in 0.25 M KI were added to the reaction mixture maintained at 2–4°. The time interval between iodine additions was 8–11 min for the first 8–9 equiv, but up to 12–16 hr was required for the last points to stabilize. The reaction is second order, and as the reactants are at very low concentrations, a decreased reaction rate would be expected near the end point of the titration (Cunningham, 1964).

Sulfhydryl Determinations in Urea. Before and after iodine oxidation, the total sulfhydryl content of the native and modified enzyme was determined in 8 M urea (recrystallized from 70% ethanol just before use). Iodination was conducted in 0.1 M KI–0.2 M sodium phosphate (pH 6.1) at a protein concentration of 4 mg/ml. After 40-min reaction at 0°, the 1-ml reaction mixture was diluted by the addition of 6 ml of 9.4 M urea containing 0.2 M sodium phosphate (pH 6.1) and 1 × 10^{-3} M 5,5′-dithiobis(2-nitrobenzoic acid) (Aldrich Chemical Co., Inc.). The absorption at 412 mμ of each sample was read within 2 min after dissolution in urea (Ellman, 1959).

Gel Filtration. The sample (approximately 5 mg of protein in 0.15–0.25 ml) was applied to a column 26.5 × 1.2 cm of Sephadex G-25 (Pharmacia) maintained at 2–3°. The column was equilibrated and eluted with 0.16 M KCl–0.2 M sodium phosphate (pH 6.1). The flow rate was maintained at 1 ml/min and fractions of 0.75 ml were collected. Each fraction was counted for ^{125}I (Nuclear-Chicago Model D55-8 well scintillation counter with Model 132 analyzer–computer) and then diluted with 0.3 or 0.5 ml of distilled water before absorbancies at 280 mμ were read.

Enzyme Inactivation. Creatine kinase was reacted for 40–60 min with 0.5–4.5 equiv of iodine/mole in 0.1 M KI–0.2 M sodium phosphate (pH 6.1) at a protein concentration of 1 mg/ml. Aliquots of the reaction mixture were diluted with glycine buffer (pH 9) and then assayed. The time lapse between dilution and assay was limited to 30 min.

Enzyme Assay. Routine assays were performed by the method of Mahowald *et al.* (1962) using a Radi-

ometer continuous-recording titrimetric pH-Stat. Results from the titrimetric method are expressed in terms of microequivalents of ATP hydrolyzed per milligram of enzyme per minute. In our laboratory, it was found that approximately 120–130 μequiv/min per mg was equivalent to 60–65 Kuby units as measured by the phosphate procedure.

Reactivation with Dithiothreitol. Reactivation experiments were performed with enzyme previously oxidized with 3–3.8 equiv of iodine/mole. Iodine oxidation was performed at a protein concentration of 2 mg/ml (0.1 M KI–0.2 M sodium phosphate, pH 6.1) for 40 min at 0°. Aliquots of 0.2 ml were combined with 0.2 ml of 0.002 M dithiothreitol and allowed 1-hr incubation before dilution with 3.6 ml of cold 0.075 M glycine buffer (pH 9). The diluted enzyme was assayed directly.

Reactivation with Dithiothreitol before and after Gel Filtration. Creatine kinase was reacted for 60 min with 3.45 equiv of ^{125}I/mole at 15 mg/ml of protein concentration (0.01 M KI–0.2 M sodium phosphate, pH 6.1). One aliquot of 0.25 ml was applied to a column of Sephadex G-25 (26.5 × 1.2 cm) and the protein peak was pooled and diluted to a concentration of 0.10 mg/ml with 0.001 M dithiothreitol–0.1 M glycine buffer (pH 9). A second aliquot of the oxidized protein was diluted in the same manner without column chromatography. A third aliquot of the iodination reaction, 0.4 ml, was incubated for 1 hr with 0.02 ml of 0.02 M dithiothreitol and then 0.25 ml of the solution was applied to the same column. The control for the experiment was a dilution of the stock enzyme solution made in the same manner plus and minus dithiothreitol.

Spectral Analysis. Enzyme was reacted with iodine for 1 hr at a protein concentration of 25 mg/ml. After gel filtration the protein solution was diluted to a concentration of 0.975 mg/ml. In order to isolate spectral differences due to intrinsic structural differences rather than concentration differences, the protein concentrations were determined by the method of Lowry *et al.* (1951) and the spectra were normalized to the same concentration. The protein solutions were scanned (against a blank containing column effluent) from 350 to 242 mμ using a Cary Model 11 recording spectrophotometer.

Equilibrium Dialysis. The oxidized enzyme was desalted on Sephadex G-25 and recovered in the buffer used for dialysis: 0.025 M Tris-HCl–0.01 M $MgCl_2$–0.125 M KCl (pH 7.22). The dialysis was performed in small plastic dialysis cells (Technilab Instruments) which were chilled on ice before assembly and filled with ice-cold solutions. One side of the cell was first filled with exactly 1 ml of dialysis buffer containing enzyme, and then the opposing side was filled with the same volume of buffer containing ADP (Schwartz BioResearch, Inc.). The two chambers were separated by a single layer of cellulose casing (Union Carbide Corp.). A control was run in the absence of protein to correct for any nonspecific binding of the nucleotide. Dialysis at 5° was continued for 20 hr with the cells mounted on a revolving stand.

The amount of nucleotide bound to the enzyme was obtained by subtracting the concentration of free nucleotide at equilibrium from the total amount added to the system. Concentration was determined using the molar absorbancy index of ADP at 259 mμ = 1.54 $10^4\ M^{-1}\ cm^{-1}$ (Bock *et al.*, 1956). The protein concentration used in the various experiments was between 7 and 8 mg per ml. The concentration of ADP was 0.002 M.

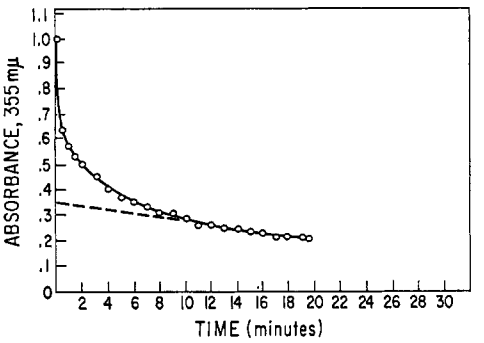

FIGURE 1: Reaction of iodine with creatine kinase, 0.2 M KI, and 0.2 M sodium phosphate (pH 6.1). See the text for additional details.

Results

Iodine Uptake Reaction. The techniques for measuring the stoichiometry of the reaction of iodine with proteins rely upon the high extinction of the triiodide anion. Thus, to determine the extent of the reaction of iodine with creatine kinase, the protein was exposed to an excess of I_3^- at pH 6.1 and 2–4°. When an aliquot of enzyme was added to a cuvet containing buffer, potassium iodide, and iodine, there was an ensuing decrease in absorbance at 355 mμ resulting from the reaction of iodine with the added protein.

Figure 1 illustrates the results of an iodine uptake reaction of creatine kinase. The decrease in absorbance at 355 mμ is plotted as a function of time. A line drawn from the "flat" or low constant slope part of the curve (usually at 10–18 min) intersects the ordinate at zero time. The optical density at this intercept is a measure of the iodine which does not react in the initial rapid reaction. Thus the difference in the initial and final iodine concentration indicates the amount of iodine consumed in rapid reaction with the added protein. The continuing slow iodine uptake probably represents side reactions of iodine with other amino acid residues.

Uptake reactions performed in this manner showed that in the presence of 15–20 equiv of iodine/mole, about 12.52 ± 0.12 equiv of iodine are consumed in reaction with 1 mole of native creatine kinase. Since this enzyme has 6 detectable sulfhydryl groups/mole in our system, the stoichiometry reduces to approximately 2 equiv of iodine/thiol group.

Creatine Kinase Modified by Reaction with Dinitrofluorobenzene. For comparison, a modified enzyme was used in an iodine uptake reaction. The two active-site sulfhydryls were blocked with 2 moles of dinitrofluorobenzene according to the conditions outlined by Mahowald *et al.* (1962). Subsequent reaction of the DNP-creatine kinase with iodine showed only 7.65 ± 0.34 equiv of iodine consumed per mole of protein. Thus it

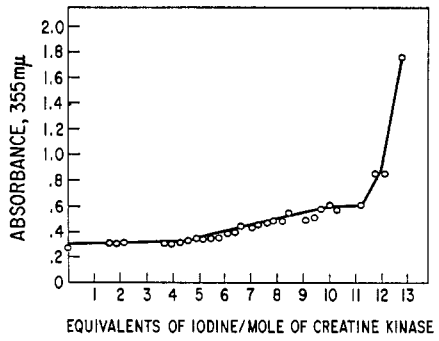

FIGURE 2: Iodine titration of creatine kinase. The 2-ml reaction mixture contained 50 mg of enzyme in 0.35 M sodium phosphate–0.2 M KI (pH 6.34).

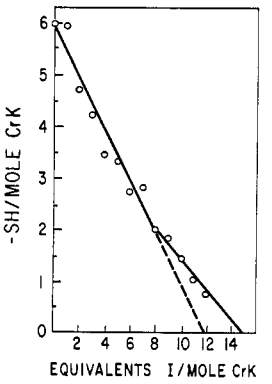

FIGURE 3: Decrease in the number of sulfhydryl groups per mole of creatine kinase with the addition of iodine; 0.1 M KI–0.2 M sodium phosphate, pH 6.1, 2–4°. See text for additional details.

appears that dinitrophenylation of two SH groups results in a decrease of approximately 4–5 equiv in the amount of iodine consumed per mole. This stoichiometry of the iodine reaction with the two "active-site" sulfhydryls can be interpreted as 2 equiv/SH group, again suggestive of oxidation to the sulfenyl level.

To further substantiate these results, the modified enzyme was examined for total sulfhydryl groups per mole and found to have 4.12 ± 0.05 SH/mole by the Ellman (1959) method, rather than the 6 found in the unmodified protein by this same method. The loss of two reactive thiol groups accounts for the decreased uptake of iodine by 4 equiv/mole.

Iodine Titration Reactions. According to the procedure of Cunningham and Nuenke (1959), the reaction of iodine with creatine kinase was examined under conditions where the concentration of protein sulfhydryl groups was 10–20 times greater than the concentration of iodine. After each iodine addition, there was a sharp increase in absorbance at 355 mμ which gradually returned to the original base level (or a stable low reading) as the iodine was consumed in the reaction. These additions were continued until there was no further decolorization, leaving a permanent color due to excess triiodide anion. This point at which there was a sharp permanent increase in absorbance was taken as the equivalence point for the reaction.

The results shown in Figure 2 indicate that the equivalence point was reached after the addition of about 12 equiv of iodine/mole of creatine kinase. Thus, although the relative concentrations of protein and iodine are different from those used in iodine uptake reactions, the stoichiometry is the same, 12 atoms of iodine/mole of enzyme. The gradual increase in residual extinction over the latter part of the reaction could be interpreted as suggesting sulfenyl iodide formation at four of the cysteinyl side chains. The rather erratic and time-dependent character of the titration curve may suggest that transient sulfenyl iodides are formed at some of the sulfhydryl groups, but that all decompose too rapidly for isolation and study.

It is possible that the increased absorbance reflects alterations in protein structure other than sulfenyl iodide formation. This explanation thus is not necessarily contradictory to other data which fail to demonstrate the formation of stable sulfenyl iodide groups.

Change in Sulfhydryl Content with Extent of Iodine Oxidation. Another approach to the definition of the chemistry of the iodine reaction was the measurement of the total number of sulfhydryl groups per mole after iodine oxidation. The results shown in Figure 3 suggest that six sulfhydryl groups are oxidized to the level of sulfenate, since there is an almost constant relationship between the thiol concentration and the extent of oxidation. Two equivalents of iodine are required for the loss of each sulfhydryl group, except perhaps at the lowest levels of protein sulfhydryl where the iodine reaction is slowest and detection is most difficult.

Studies with $^{125}I_2$-Oxidized Creatine Kinase. The stoichiometry of the reaction of iodine with this enzyme indicates oxidation to the level of sulfenate. The titration curve of Figure 2 also provided suggestive evidence for at least a transient sulfenyl iodide and it was of interest to test directly whether this was actually the case with creatine kinase.

A stable sulfenyl iodide group has been described in studies of TMV (Fraenkel-Conrat, 1955), but the sulfenyl iodide groups of β-lactoglobulin and ovalbumin do not survive dialysis and lyophilization procedures (Cunningham and Nuenke, 1960). In consideration of the lability of the sulfenyl iodide groups, the iodine-oxidized creatine kinase was analyzed under conditions selected to minimize protein sulfenyl iodide decomposition.

The enzyme was oxidized using labeled iodine, $^{125}I_2$, so that if any protein sulfenyl iodides were formed, radioactive iodine would remain bound to the protein. The oxidized protein prepared with the $^{125}I_2$ was separated from the radioactive reaction mixture by gel filtration. The following experiments represent a series of attempts to measure quantitatively the radioactive iodine associated with the iodine-oxidized enzyme.

Creatine kinase was reacted with 6.0 equiv of labeled iodine/mole of protein. An aliquot of the oxidized protein was applied to a column of Sephadex G-25 and eluted under the conditions described previously. Other aliquots were preincubated with a fivefold molar excess of Tapazole (1-methyl-2-mercaptoimidazole) or mercaptoethanol. The molar excess of the latter compounds was calculated on the basis of the maximum protein sul-

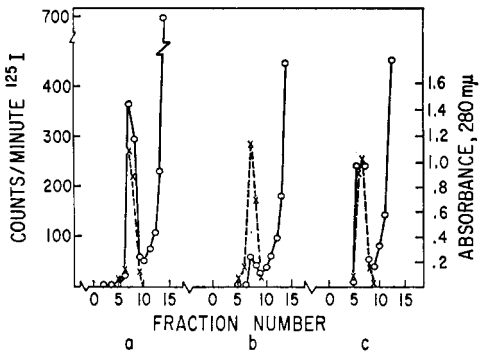

FIGURE 4: Gel filtration of modified creatine kinase on Sephadex G-25, 0.016 M KCl–0.2 M sodium phosphate, pH 6.1, 2–3°. (a) Enzyme reacted with 6.9 equiv of iodine/mole. (b) Enzyme reacted with 6.9 equiv of iodine/mole followed by incubation with a fivefold molar excess of Tapazole. (c) Enzyme reacted with 6.9 equiv of iodine/mole followed by incubation with a fivefold molar excess of mercaptoethanol. (O——O) Counts per minute and (X- - - -X) absorbance 280 mμ.

fenyl iodide concentration which might result from stoichiometric reaction with the added iodine according to the equation

$$RSH + I_2 \longrightarrow RSI + H^+ + I^-$$

After preincubation, aliquots were applied to the Sephadex column and eluted in the same manner. These results are shown in Figure 4.

Assuming stoichiometric reaction of all the iodine to form sulfenyl iodide, half the amount of iodine would remain with the protein. However, only 1.3% of the theoretical number of counts was found associated with the protein (Figure 4A). Pretreatment with Tapazole removed all but 15% of this small amount (Figure 4B) but pretreatment with mercaptoethanol was much less effective (Figure 4C).

Stoichiometry of Enzyme Inactivation. The results shown in Figure 5 indicate that only 4 equiv of iodine/mole is required to inactivate native creatine kinase. The inactivation appears to be linear until approximately 3.5 equiv of iodine/mole has been added. Thereafter the remaining enzyme activity is extremely low and difficult to measure accurately. However, a straight line drawn through the other points extrapolates to a value of approximately 4 equiv of iodine/mole required to abolish enzyme activity completely.

Only two thiol groups of creatine kinase react with most sulfhydryl reagents in the absence of denaturants. These two reactive cysteinyl residues have been shown to be essential for the activity of the enzyme (Mahowald *et al.*, 1962). Sulfhydryl determinations on the native enzyme, performed by the Ellman procedure (Ellman, 1959), measure only these active-site cysteinyl residues. This iodination study was performed at a higher protein concentration allowing both sulfhydryl determinations and activity measurements to be made on the same reaction mixtures.

The loss of enzyme activity appears to parallel the loss of the two active-site sulfhydryl groups as shown in

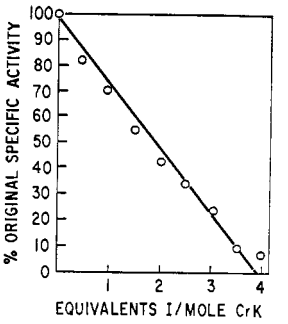

FIGURE 5: Loss of enzyme activity with iodine oxidation; 0.1 M KI–0.2 M sodium phosphate (pH 6.1), protein concentration 1 mg/ml.

Figure 6. Thus these two groups exhibit a much higher reactivity toward iodine than the other sulfhydryl groups of this protein (Figure 3).

Reactivation of the Partially Oxidized Enzyme. Preliminary experiments showed that the partially oxidized enzyme could be reactivated. However, if the protein were allowed to react with more than 4 equiv of iodine/mole, reactivation failed. These reactivation studies were performed with reducing agents which might convert protein SI and SOH groups back into thiol groups (Cunningham and Nuenke, 1960).

In order to better define the chemical nature of the modified cysteinyl group after gel filtration, reactivation experiments were conducted on the oxidized enzyme before and after column chromatography. If gel filtration resulted in alterations in the cysteinyl residues, *e.g.*, further oxidation to SO_2H, then such changes might be manifested by differences in reactivation.

Tapazole is one of a group of compounds containing the thiourea structure (N(H)C(=S)) which is especially reactive with protein sulfenyl iodides (Cunningham, 1964). If iodine oxidation of creatine kinase converted some thiol groups into sulfenyl iodides, then Tapazole might be expected to be a more effective reactivating compound than mercaptoethanol. However, a complicating feature of such an hypothesis is the likelihood of complex disulfide-exchange reactions leading to variable amounts of free enzyme sulfhydryl groups. The results of this experiment are shown in Table I.

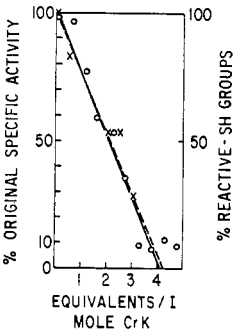

FIGURE 6: Decrease in enzyme activity and reactivity of active-site sulfhydryl groups upon addition of iodine; 0.1 M KI–sodium phosphate (pH 6.1), protein concentration 4 mg/ml, and 1.78 thiol groups/mole. (O——O) Enzyme activity and (X- - - -X) sulfhydryl content.

TABLE I: Reactivation of Iodine Oxidized Enzyme before and after Gel Filtration. Comparison of Mercaptoethanol and Tapazole.

Enzyme Sample	Sp Act.[a]	% of Sp Act. of Control
Control	103.8 ± 1.41	100
Control + 3.54 equiv of I_2/mole	24.95 ± 3.2	24
Control + 3.54 equiv of I_2/mole + Tapazole	33.27 ± 0.47	32
Control + 3.54 equiv of I_2/mole + mercaptoethanol	101.9 ± 2.4	98.4
Gel filtered, oxidized enzyme	23.6 ± 0.5	22.7
Gel filtered + Tapazole	29.3 ± 0.69	28.3
Gel filtered + mercaptoethanol	67.0 ± 0.3	65.3

[a] Mean numerical average plus and minus standard deviation from three assays.

Several dithiothreitol reactivation studies were performed before and after gel filtration using enzyme oxidized with ^{125}I to allow counting and reactivation measurements to be made with enzyme from a single iodination reaction. The effect upon enzyme activity is shown in Table II.

The Sephadex chromatography procedure did not change either the specific activity of the oxidized enzyme, or the extent of reactivation when performed under optimum conditions where dithiothreitol is used as the reactivating agent (shown in Table II). Thus gel filtration of the oxidized protein does not appear to greatly alter the chemical nature of the modified thiol groups.

Physical Studies of the Oxidized Enzyme. Following gel filtration and correction for concentration differences, there were no differences between the typical protein ultraviolet absorbtion spectra of the native enzyme and that reacted with 4 equiv of iodine/mole.

Equilibrium dialysis was employed to compare the substrate binding capacity of the native and oxidized creatine kinase. Dissociation constants for $MgADP^-$ were determined for the control, $K_D = 2.47 \pm 0.2 \times 10^{-4}$, and iodine-oxidized enzyme, $K_D = 3.76 \pm 0.2 \times 10^{-4}$. Thus the inactive enzyme still binds the substrate almost as well as the native enzyme.

Discussion

Except perhaps for the studies of serum albumin (Hughes and Straessle, 1950), this present study represents the first clear demonstration of the oxidation of structurally restrained sulfhydryl groups to the level of sulfenic acid (Bruice and Sayigh, 1959). In addition to the two active-site sulfhydryl groups, four of the thiol groups

TABLE II: Dithiothreitol-Catalyzed Reactivation of Iodine-Oxidized Enzyme before and after Gel Filtration.

Sample	Sp Act. (μequiv/min per mg)	% of the Sp Act. of the Control
Control	119	100
Control + dithiothreitol	123.4	103.5
Creatine kinase + 3.45 equiv of I_2/mole	28.3	23.8
Creatine kinase + 3.45 equiv of I_2/mole + dithiothreitol	95.4	80
Gel filtered oxidized Creatine kinase	31.4	26
Gel filtered + dithiothreitol	98.8	83

of creatine kinase, which ordinarily are unreactive toward sulfhydryl reagents, react with stoichiometric amounts of iodine. However, the remaining two cysteinyl residues are inert under these conditions.

Several experimental approaches revealed that 2 equiv of iodine was consumed in reaction with each of the six thiol residues, indicating oxidation to the level of sulfenate. As shown in the following reactions, this stoichiometry could result in the formation of either sulfenic acid (eq 1) or sulfenyl iodide (eq 2)

$$RSH + I_2 \xrightarrow{+H_2O} RSOH + 2H^+ + 2I^- \quad (1)$$

$$RSH + I_2 \longrightarrow R{-}SI + H^+ + I^- \quad (2)$$

moieties. Further definition of the chemistry of this oxidation came from reactivation experiments, physical studies, and labeling experiments with radioactive iodine.

All the experiments using radioactive iodine consistently showed only a very small amount of iodine remaining with the protein peak, no more than 1–5% of the theoretical amount. These values are too low to account for stoichiometric sulfenyl iodide formation, but they do not rule out the possibility of sulfenyl iodide as a transient intermediate. This transient sulfenyl iodide group, upon subsequent hydrolysis, yields the final reaction product, a protein sulfenic acid group or some form of S^+. Further direct chemical studies of the oxidized protein will be necessary to answer this question.

Almost all of the radioactivity remaining with the protein can be displaced by incubation with Tapazole, a reagent shown to react readily with sulfenyl iodide groups of other proteins, releasing bound iodine. The reversibility of this very small amount of bound iodine suggests the presence of a small amount of sulfenyl iodide and also serves to rule out noncovalent binding of the iodine to the protein as an explanation for the small amount of radioactive iodine present.

During iodine oxidation, the loss of catalytic activity parallels the loss of the two active-site sulfhydryl

groups. Thus the conversion of these free thiol groups into sulfenyl iodides or sulfenates is sufficient to destroy enzyme activity. This result agrees with similar studies in which modification of these same two cysteinyl residues with iodoacetic acid or dinitrofluorobenzene abolished all biological activity (Mahowald *et al.*, 1962). Reactivation of the inactive enzyme probably occurs in the following manner with the intermediate formation of mixed disulfides.

$$PSOH + RSH \longrightarrow PSSR + H_2O \text{ or}$$

$$PSI + RSH \longrightarrow PSSR + H^+ + I^-, \text{ then}$$

$$PSSR + RSH \longrightarrow RSSR + {}'PSH$$

Iodine oxidation of cysteinyl side chains could produce general conformational changes which would be reflected in altered physical properties. However, the almost complete reactivation of the enzyme after exposure to 4 equiv of iodine/mole suggests that the inactive protein had not undergone any gross changes in tertiary structure.

Comparison of the substrate binding capacity of the native enzyme and the partially oxidized, inactive enzyme indicated that the inactive enzyme still binds the substrate, although not as well as the native enzyme. This decrease in the strength of interaction between the oxidized enzyme and the substrate is not great enough to account for the observed loss of approximately 90% of the catalytic activity under our assay conditions. Thus the inactivation cannot be ascribed solely to decreased ability to bind ADP, and a more active role for the sulfhydryl group in the catalytic mechanism must be anticipated.

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