Reaction of Reactive Sulfhydryl Groups of Creatine Kinase with Dansyl Chloride*

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ABSTRACT: The fluorescent chromophore dansyl chloride, 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNSCl), in limited molar excess reacts with creatine kinase in 0.1 M $NaHCO_3$, pH 8.5–9.0, at room temperature to produce a completely inactive enzyme. Inactivation is correlated with the loss of two reactive sulfhydryl groups in the enzyme. None of the fluorescent reagent remains attached to the protein after inactivation.

Although a model reaction of cysteine in 0.1 M $NaHCO_3$, pH 8.5–9.0, with DNSCl proceeds with 100% conversion into cystine, disulfide formation does not occur on reaction of the enzyme with this compound. Ultracentrifugation studies reveal no difference in the sedimentation velocities of native and DNS-treated enzyme, thus eliminating the possibility of intermolecular disulfide formation. At pH 6.1 in 0.1 M NaH_2PO_4 buffer on ice, a twofold excess of DNSCl is sufficient to inhibit creatine kinase. There is a linear relationship between the loss of activity and the disappearance of two reactive sulfhydryls. This stoichiometry does not seem consistent with disulfide formation. Under these conditions, furthermore, incubation of the enzyme with dithiothreitol following treatment with DNSCl regenerates the activity. Reactivation presumably takes place *via* a mixed disulfide exchange to restore the original sulfhydryl group. The stoichiometry of the reaction at pH 6.1, the absence of DNS associated with the enzyme, and the regeneration of the enzyme activity with excess thiols seem best interpreted as reflecting oxidation of the reactive sulfhydryls to the sulfenyl level, presumably sulfenic acid, by the breakdown of a labile thiolsulfonate intermediate. At pH 6.1 the sulfenyl state is apparently stabilized by the microenvironment of the cysteine residues in the active site while at pH 8.5–9.0 oxidation proceeds to the less easily reversible sulfinic and/or sulfonic acid oxidation states by an as yet undetermined mechanism.

Little has been done in previous investigations to characterize complexes of proteins and enzymes with fluorescent chromophores. With the advent of fluorescence spectroscopy knowledge of the loci of conjugation of the fluorescent probe to the protein has become increasingly important for an accurate interpretation of fluorescence data.

One of the most commonly used fluorescent reagents is the sulfonyl chloride, DNSCl.[1] Weber (1952) suggested that DNSCl reacts primarily with amino groups of ovalbumin and serum albumin to form sulfonamide linkages. DNSCl has also been reported to modify a reactive histidine (Hartley and Massey, 1956) or a reactive serine (Gold, 1965) at the active site of chymotrypsin. Hartley and Massey (1956) further demonstrated that DNSCl could react with amino, phenol, sulfhydryl, and imidazole groups of free amino acids. They concluded that the amino derivatives are stable to acid and alkali, that the modified phenols are stable to acid hydrolysis, that the sulfhydryl derivatives are acid labile but stable in the pH range 5–10, and that the DNS–imidazole complex is unstable in both acid and alkali.

Kassab *et al.* (1968) reported that the reaction of arginine kinase, an enzyme similar in many ways to creatine kinase, with a 25-fold excess of DNSCl at pH 8–9 effected the loss of sulfhydryls and enzymatic activity and resulted in the incorporation of 1 mole of DNSCl into a reactive lysine in the enzyme. These results were interpreted to mean that modification of the reactive lysine destroyed the conformation of the active site and allowed formation of a disulfide bridge which could be easily reduced with dithiothreitol. In the same paper these authors showed that DNSCl reacted with amino groups in creatine kinase after the sulfhydryl groups had been blocked with tetrathionate. However, no comment was made concerning the reaction of the DNSCl with the sulfhydryl groups in this enzyme.

Since many changes in the structure and function of proteins have been attributed to the oxidation of thiols and disulfides and since opinions differ regarding the intermediate compounds in oxidation–reduction equilibrium with thiols and disulfides, the present investigation was undertaken to determine the mechanism whereby the sulfonyl chloride, DNSCl, modified sulfhydryls in aqueous media and to learn more about the actual oxidation states of sulfur necessary for maintaining activity and conformation in creatine kinase. The direct correlation between intact sulfhydryls and biological activity in this enzyme afforded a convenient method for monitoring these effects.

Materials and Methods

Creatine Kinase. Creatine kinase was isolated from frozen rabbit muscle by procedure B of Kuby *et al.* (1954) and was

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[1] Abbreviations used are: DNSCl, 1-dimethylaminonaphthalene-5-sulfonyl chloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol.

crystallized two times according to the improved method of Mahowald *et al.* (1962). The twice-crystallized protein had an activity of 110–130 μequiv of ATP hydrolyzed/min per mg of protein by the pH-Stat method of Mahowald *et al.* (1962) and moved as a homogeneous peak in the analytical ultracentrifuge. Protein concentrations were determined spectrophotometrically at 280 mμ using $\epsilon_{1cm}^{1\%}$ 8.8 (Noda *et al.*, 1954; Kuby and Noltman, 1962).

DNSCl Solutions. The DNSCl used in these experiments was obtained in acetone solutions (100 mg/ml) from Pierce Chemical Co. (specially purified grade, hypovial sealed under nitrogen). Since dansylsulfonic acid, the hydrolysis breakdown product of DNSCl, is insoluble in acetone, it is easily detected in these solutions as white crystals. No solution of DNSCl which was discolored or which contained such a precipitate was used. For each experiment an aliquot of the concentrated DNSCl was removed with a syringe, quickly filtered, and diluted with dry acetone immediately before use. During the course of the experiment these stock solutions were kept tightly stoppered and wrapped in Parafilm to minimize concentration changes due to evaporation. The concentration of DNSCl was determined after dilution with ethanol from the absorption at 369 mμ assuming a molar extinction coefficient of 3.73×10^3 M^{-1} cm^{-1} (Weber, 1952).

Reaction of Creatine Kinase with DNSCl. Creatine kinase (1–4 mg/ml) which had been dialyzed exhaustively against 0.1 M $NaHCO_3$ was treated with acetone solutions of DNSCl in limited molar ratios at room temperature. At a given time aliquots were removed and diluted with 0.001 M glycine, pH 9.0, for assay of enzymatic activity. The acetone concentration never exceeded 9.1% but caused the pH of the reaction mixture to increase from pH 8.3 to about pH 9 as measured by a glass electrode.

The same reaction was also done in 0.1 M NaH_2PO_4, pH 6.1, at 4°. In these experiments the creatine kinase was not dialyzed against buffer prior to the reaction.

Determinations of the Sulfhydryl Content of Creatine Kinase. At the same time that aliquots were removed from the reaction mixture for assay of enzyme activity, aliquots were also removed for reaction with DTNB (Aldrich Chemical Co., Inc.) after the method of Ellman (1959). To 0.5–0.8 ml of 0.05 M NaH_2PO_4 buffer, pH 8.0, was added 0.8–0.2 ml of the reaction mixture containing 1–4 mg of native or modified creatine kinase per ml and 10–20 μl of a 0.01 M solution of DTNB in 0.05 M NaH_2PO_4 buffer, pH 7.0. The reaction was allowed to proceed at room temperature for 2–15 min. The absorption at 412 mμ was read, and the concentration of free sulfhydryls was determined from a standard curve prepared under similar conditions with reduced glutathione (Sigma Chemical Co.) using a molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}. This method gave a value of 1.8–2.2 moles of reactive SH per mole of creatine kinase.

Sulfhydryl Determinations in Urea. Native or DNS-treated creatine kinase was denatured with 8 M urea for 5 min before DTNB was added. Absorption at 412 mμ was read within 2–5 min.

Gel Filtration of DNS-Treated Creatine Kinase and Quantitation of the Amount of DNS Bound to the Enzyme. To isolate DNS-treated creatine kinase from the reaction mixture, 1.0 ml of the reaction mixture containing creatine kinase at a concentration of 10–15 mg/ml was passed over a 1 × 25 cm Sephadex G-25 (Pharmacia) column in 0.1 M $NaHCO_3$, pH8 .5–9.0, at room temperature or over a 1 × 12 cm. Sephadex G-25 column in 0.1 M NaH_2PO_4, pH 6.1, at 4°. Approximately 1.0-ml fractions were collected. The protein peak and the peak containing the breakdown products of DNSCl could be located spectrophotometrically at 280 and 312 mμ, respectively. Brief ultraviolet illumination of the tubes with a Mineralite lamp could also be used to detect the fluorescence of the breakdown products of DNSCl and the DNS–protein when the latter was labeled.

The addition of DNSCl to the protein solution resulted initially in a turpid reaction mixture due to the insolubility of DNSCl in aqueous solutions. However, the reaction mixtures cleared before they were chromatographed on Sephadex indicating reaction or hydrolysis of DNSCl to form the soluble sodium salts of the dansyl acids. Spectra of the breakdown products in the effluent repeatedly showed a maximum at 306–310 mμ, a value close to 312 mμ, the maximum for dansylsulfonic acid.

The amount of DNS bound to the enzyme was determined in two ways after gel filtration: (1) by reading the absorption of the protein peak at 340 mμ, using an extinction coefficient of 4.3×10^3 M^{-1} cm^{-1} (Weber, 1952) and determining the concentration of the protein from the absorption at 280 mμ; (2) by calculating the concentration of breakdown products of DNSCl in the effluent from the optical density at 312 mμ using the extinction coefficient of 4.81×10^3 M^{-1} cm^{-1} reported for DNS-sulfonic acid (Gray, 1964) and subtracting the amount of breakdown products from the amount of DNSCl originally present in 1.0 ml of the reaction mixture.

Reactivation of Modified Creatine Kinase with Mercaptoethanol and Dithiothreitol. After modification of the sulfhydryls of creatine kinase with DNSCl, aliquots of the reaction mixture were either diluted 1:1 with 0.2 M mercaptoethanol in buffer or water to give a final mercaptoethanol concentration of 0.1 M or diluted with 0.001 M glycine, pH 9.0, containing 0.01 M dithiothreitol to a final creatine kinase concentration of 0.08–0.1 mg/ml. The reaction mixtures were incubated on ice for 30–60 min and then assayed for enzymatic activity. Aliquots of the mercaptoethanol incubation mixture were further diluted with 0.001 M glycine, pH 9.0, before assay. Aliquots for enzymatic assay were removed directly from the tubes containing dithiothreitol. In some experiments reactivation was carried out after gel filtration of the reaction mixture.

Sedimentation Velocity Measurements of Native and DNS-Treated Creatine Kinase. Sedimentation constants for native and DNS-treated creatine kinase were determined from measurements obtained in the Spinco Model E analytical ultracentrifuge. The protein concentration ranged from 3.4 to 7.6 mg per ml. All runs were made at 59,780 rpm in 0.1 M Tris–0.15 M NaCl, pH 7.86, buffer. Temperatures ranged from 19 to 22° for different runs. Pictures were taken at 16-min intervals with a 7-sec exposure and a bar angle of 50–60°.

Model Reaction of Cysteine with DNSCl. A freshly prepared solution of cysteine in nitrogen-flushed 0.1 M $NaHCO_3$ was treated with acetone solutions of DNSCl in a ratio of 2 moles of cysteine per mole of DNSCl at room temperature for 5 min. A control without DNSCl but with acetone was run under the same conditions. An aliquot of the reaction mixture was then diluted with 0.2 M sodium citrate buffer, pH 2.2, and analyzed on a Model 120 Beckman Spinco automatic amino acid analyzer.

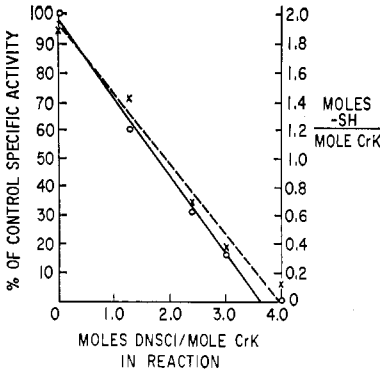

FIGURE 1: Decrease in the enzyme activity and active site sulfhydryl groups of creatine kinase on reaction with DNSCl, pH 8.5–9.0; (O—O) activity, (×---×) sulfhydryl content; the dansylation reaction was performed at room temperature in 0.1 M $NaHCO_3$, pH 8.5–9.0, at a protein concentration of 3–4 mg/ml. Aliquots were removed for assay of enzymatic activity and for reaction with DTNB.

TABLE I: Measurement of Sulfhydryl Groups[a] in DNS-Treated Creatine Kinase in the Presence and Absence of Urea.

Sample	Moles of SH/Mole of CrK[b]	% of Control SH Remaining
Control	1.88 (±0.05)	100
CrK + 4 moles of DNSCl	0.031 (±0.005)	1.6
Control + urea[c]	5.93 (±0.91)	100
CrK + 4 moles of DNSCl + urea[c]	4.19 (±0.31)	70.1

[a] Sulfhydryl content was determined with DTNB as described in the text. [b] Each value represents the mean numerical average and the maximum deviation of two determinations. [c] Urea was added after the treatment of the enzyme with DNSCl or acetone and before the addition of DTNB to the reaction mixture. Values were corrected for dilution due to addition of solid urea to 8 M.

Results

Reaction of Creatine Kinase with DNSCl at pH 8.5–9.0. When creatine kinase in 0.1 M $NaHCO_3$, pH 8.5–9.0, was treated with a 4 molar excess of DNSCl at room temperature, complete inhibition of the enzyme activity occurred. However, little, if any, of the DNSCl remained attached to the protein by the criteria of absorption of the protein fraction at 340 mμ, the sulfonamide absorption maximum, or analysis of the breakdown products of DNSCl in the effluent. Furthermore, examination of the ultraviolet spectrum of this DNS-treated protein failed to reveal an absorption maximum which might reflect the reaction of the DNSCl with some other group in the protein. It was therefore concluded that the DNSCl initially attacked an important group, different from lysine, in or near the active site of creatine kinase to form a labile intermediate. In the spontaneous breakdown of the intermediate the reactive side chain was modified so as to yield an inactive enzyme.

Effect of the DNSCl Reaction on the Sulfhydryl Groups in Creatine Kinase. Since the reactivity of the "active site" sulfhydryls of this enzyme is so well documented, attention was focused on the reaction of DNSCl with cysteine residues. When creatine kinase in 0.1 M $NaHCO_3$ was titrated with limited molar ratios of DNSCl and subsequently analyzed for enzymatic activity as well as for free sulfhydryls by the method of Ellman (1959), the results shown in Figure 1 were obtained. The disappearance of two sulfhydryls paralleled the disappearance of the enzymatic activity, thereby substantiating the hypothesis that the sulfhydryls were involved in the inhibition of activity with DNSCl.

The stoichiometry observed in this reaction repeatedly indicated that about 4 moles of DNSCl was required for complete inhibition instead of the expected 2. These results were the same whether the reaction was run for 5 min or for 1 hr.

It seemed probable, however, that much of the DNSCl was being rapidly and spontaneously hydrolyzed in the bicarbonate buffer at room temperature and that the actual consumption of DNSCl in this reaction was 2 moles per mole of enzyme. This assumption was based on Gray's observation (1964) that at 10^{-6} M dissolved DNSCl has a half-life of about 5 min at neutral pH.

Evidence from the literature indicates that there are a total of eight half-cystine residues per mole of creatine kinase (Yue *et al.*, 1967; Bayley and Thomson, 1967). Of these, two are the especially reactive, active site sulfhydryl groups, four are usually unreactive except under denaturing conditions, and two are extremely resistant to chemical modification (Noltmann *et al.*, 1962) and have been detected only by amino acid analysis of the denatured, reduced, carboxymethylated enzyme. The data of Figure 1 suggest that inactivation with DNSCl is due to modification of the active site sulfhydryls. To substantiate this creatine kinase was first treated with a 4 molar excess of DNSCl and then with DTNB in the presence and absence of urea. Controls containing acetone but no DNSCl were also run under the same conditions. These results, summarized in Table I, demonstrate that no more than 2 sulfhydryls were modified by the DNSCl reaction and that these are the "active site" sulfhydryls which are exposed in the native enzyme. Reaction of the DNS-treated denatured enzyme with DTNB shows that the four sulfhydryls which are unreactive in the native enzyme are still intact after the DNSCl reaction.

Reaction of Creatine Kinase with Dansylsulfonic Acid. To eliminate the possibility that inhibition was due to steric hindrance produced by hydrophobic binding of the naphthalene moiety in or near the "active site" sulfhydryl rather than to the actual sulfonylation of the sulfhydryl by the DNSCl molecule, creatine kinase in 0.1 M $NaHCO_3$ was treated for 5 min at room temperature with a 6 molar excess of dansylsulfonic acid prepared in this laboratory by the hydrolysis of DNSCl. Since no inhibition of activity was observed under these conditions, it was concluded that chemical modification of the sulfhydryl was required for inactivation.

Effect of Substrates on the Reaction of Creatine Kinase with DNSCl. The rate of inhibition of creatine kinase by iodoacetamide (Watts and Rabin, 1962; Watts, 1963) is decreased

in the presence of equilibrium mixtures of substrates of the enzyme (MgATP and creatine and MgADP and P-creatine). In addition, MgADP and creatine, although nonfunctional, decrease the rate of inhibition of the enzyme by iodoacetate, iodoacetamide, *p*-nitrophenylacetate, and of digestion by trypsin (Watts, 1963; Watts and Rabin, 1962; O'Sullivan and Cohn, 1966; O'Sullivan *et al.*, 1966; Lui and Cunningham, 1966; Jacobs and Cunningham, 1968). It seemed possible that substrates might also afford protection in the reaction of the enzyme with DNSCl.

When a mixture of the enzyme and its substrates was treated with DNSCl in a 3 molar excess for 15 min at room temperature the results shown in Table II were obtained. In the absence of substrates a 3 molar excess of DNSCl inhibited the enzyme to 11.8% of the control activity. Mg-creatine showed no protection against inhibition by DNSCl. MgADP, on the other hand, showed slight protection which might be attributed to an induced conformational change on the binding of this substrate as proposed by O'Sullivan *et al.* (1966) and Lui and Cunningham (1966). Maximal, but not complete, protection was demonstrated with the complete substrate mixture. The failure of substrates to offer greater protection under these conditions is apparently due to the very rapid and irreversible reaction of DNSCl with the enzyme.

Disulfide Formation on Reaction of DNSCl with Model Thiols. In an attempt to determine a plausible mechanism for the reaction of DNSCl with the sulfhydryls of creatine kinase, a model reaction was run with DNSCl and cysteine under the same conditions used for the reaction with the protein. In the presence of excess thiol the reaction of a thiol with a sulfonyl chloride would be expected to occur according to eq 1 and 2 (Gibson *et al.*, 1925; Parsons *et al.*, 1965). These reactions indicate a stoichiometry of 1 mole of sulfonyl chloride per 2 moles of thiol to produce a disulfide from the thiol and sulfinic acid from the sulfonyl chloride.

$$ArSO_2Cl + RSH \rightleftharpoons ArSO_2SR + H^+ + Cl^- \quad (1)$$

$$ArSO_2SR + RSH \rightleftharpoons RSSR + ArSO_2H \quad (2)$$

When cysteine was treated with DNSCl under the conditions previously described, the reaction proceeded with 100% conversion into cystine as measured by the ninhydrin reaction of fractions off Dowex 50 on the Beckman Spinco automatic amino acid analyzer. The control with acetone but no DNSCl run under the same conditions showed only about 7% conversion into cystine. The results of this oxidation were consistent with those expected from eq 1 and 2.

Absence of Disulfide Formation in Creating Kinase at pH 8.5–9.0. Yue *et al.* (1967) reported that a preparation of three-times-crystallized creatine kinase which had been lyophilized in 0.01 M glycine, pH 9.0, and stored at −10° for 2 years showed a trace of a heavier sedimenting component with a $s_{20,w}$ of about 6.8. This heavier material disappeared after addition of 0.1 M mercaptoethanol to the enzyme. The authors interpreted this observation as possible intermolecular disulfide formation. If an intermolecular disulfide bridge were in fact formed under those conditions, then DNSCl, which can catalyze the formation of disulfides from excess thiol, might form similar aggregates with this protein.

To investigate the possibility of intermolecular disulfide formation in DNS-treated creatine kinase, creatine kinase (7.2 mg/ml) in 0.1 M $NaHCO_3$ was treated with a 4 molar excess of DNSCl in acetone for 15 min at room temperature. The DNS-treated protein was then dialyzed exhaustively against 0.1 M Tris–0.15 M NaCl buffer, pH 7.86, at 4°. Ultracentrifugations were performed in a Model E Spinco analytical ultracentrifuge at 59,780 rpm at 19°. Untreated twice-crystallized enzyme was used under the same conditions at 22°. In both cases the protein ran as a single symmetrical peak. The $s_{20,w}$ obtained for the DNS-treated enzyme was 5.12–5.45 × 10^{-13} sec while that obtained for native creatine kinase was 5.12–5.38 × 10^{-13} sec. These values are in close agreement with the values of 5.11–5.31 reported by Yue *et al.* (1967) for native creatine kinase.

TABLE II: Substrate Protection[a] of Creatine Kinase toward Reactions with DNSCl.[b]

Sample	Activity, μequiv of ATP/min per mg of Protein	Activity, % of Control
Control	98.7	100
CrK + DNSCl[c]	11.6	11.8
CrK, MgADP + DNSCl	23.6	23.9
CrK, Mg, creatine + DNSCl	9.3	9.4
CrK, MgADP, creatine + DNSCl	34.0	34.5

[a] Final concentrations of components of the reaction mixture were as follows: ADP = 5.5 × 10^{-3} M; creatine = 7.3 × 10^{-2} M; Mg^{2+} = 1.4 × 10^{-2} M; CrK = 3.3 × 10^{-5} M. [b] Buffer is 0.1 M $NaHCO_3$, pH 8.5–9.0. [c] Three moles of DNSCl per mole of Crk was added to the reaction mixture.

The above experiment ruled out intermolecular disulfide formation, and the possibility of intramolecular disulfide formation was substantially eliminated by the sulfhydryl balance studies of Figure 1 and Table I. In order to investigate further the products of the reaction, creatine kinase was partially oxidized with a 2.1 molar excess of DNSCl for about 1 min at room temperature and an aliquot was diluted immediately for assay. At the same time an aliquot of the reaction mixture was removed and allowed to incubate in 0.1 M mercaptoethanol for about 45 min before assay. A comparison of of the activities of these two aliquots with that of a control run under the same conditions is presented in Table III. Since either an intermolecular or an intramolecular disulfide should be easily reduced under these conditions, the failure of added mercaptoethanol to restore enzymatic activity suggested oxidation of the sulfhydryls by DNSCl to some irreversible level beyond the disulfide, presumably to the sulfinic and/or sulfonic acid levels. This oxidation presumably occurred by the breakdown of a labile thiolsulfonate intermediate according to eq 3 and 4 (Parker and Kharasch, 1959).

$$\text{Protein–S–SO}_2\text{Ar} + H_2O \longrightarrow \text{Protein–SOH} + ArSO_2H \quad (3)$$

$$\text{Protein–SOH} \xrightarrow[OH^-]{[O]} \text{Protein–SO}_2\text{H} \xrightarrow[OH^-]{[O]} \text{Protein–SO}_3\text{H} \quad (4)$$

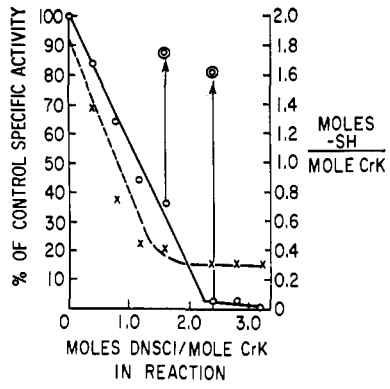

FIGURE 2: Decrease in enzyme activity and active site sulfhydryl groups on reaction with DNSCl, pH 6.1, and reactivation with dithiothreitol; (O—O) activity, (×---×) sulfhydryl content; dansylation was performed in 0.1 M NaH_2PO_4, pH 6.1, for 5 min at 4° at a protein concentration of 3–4 mg/ml. Aliquots of the reaction mixture were removed for assay of enzymatic activity, for reaction with DTNB, and for incubation on ice with 0.01 M DTT. After about 1 hr the incubation mixture was assayed for enzymatic activity.

Protein–SOH has also been postulated as an intermediate in the oxidation of creatine kinase with I_3^- at pH 6.1 and 2–4° (Trundle and Cunningham, 1969). Incubation of the oxidized protein with thiols in that study resulted in considerable restoration of enzymatic activity, probably because of the lower pH and temperature of the oxidation.

Reaction of Creatine Kinase with DNSCl at pH 6.1. Since the loss of activity upon iodine oxidation of creatine kinase had exhibited such extensive reversibility with dithiothreitol, the reaction of creatine kinase with DNSCl was examined under similar conditions in attempts to learn more about sulfur intermediates in this reaction. DNSCl in acetone was added in limited molar ratios to a series of tubes containing creatine kinase in 0.1 M NaH_2PO_4 at pH 6.1 at 4°. After 5 min aliquots were assayed. Other aliquots were diluted with glycine containing 0.01 M dithiothreitol and incubated on ice for an additional 30 min before assay. A third series of aliquots was treated with DTNB and read at 412 mμ to quantitate the amount of free sulfhydryls remaining. These results are shown in Figure 2. As observed in the reaction at pH 9.0, the disappearance of sulfhydryls was coincident with the loss of enzymatic activity. However, in contrast to the previous experiments which showed a maximum of 10% increase in activity after incubation with mercaptoethanol or dithiothreitol, the intermediate produced at pH 6.1 could be reactivated from 2% of the control activity to 70%. Furthermore, the stoichiometry at pH 6.1 was close to 2 moles of DNSCl per mole of creatine kinase, the theoretical ratio expected for oxidation to the sulfenyl level.

Since 4 moles of DNSCl is required to completely inhibit creatine kinase activity in bicarbonate buffer, pH 9.0, at room temperature while only 2 moles is required for inhibition in phosphate buffer, pH 6.1, at 4°, it is apparent that the rate of the reaction under the conditions at pH 6.1 is much greater than at pH 9.0 and/or that the rate of hydrolysis of DNSCl at the lower temperature and lower pH is significantly decreased. The evaluation of the stoichiometry at pH 6.1 is further complicated by the fact that the sulfhydryl content as determined by Ellman's reaction does not decrease below about 15% of the control. The stoichiometry observed for inhibition of the activity of creatine kinase at pH 6.1 together with the results from earlier iodine oxidation studies of creatine kinase (Trundle and Cunningham, 1969) under similar conditions strongly support the argument of a sulfenyl instead of a disulfide intermediate.

To exclude the possibility that the reversible intermediate in this reaction at pH 6.1 might be the thiolsulfonate instead

TABLE III: Reaction of Creatine Kinase with DNSCl, pH 8.5–9.0, Followed by Reactivation with 0.1 M Mercaptoethanol.[a]

Sample	Activity,[b] μequiv of ATP/min per mg of Protein	Activity, % of Control
Control	105.2 (±1.2)	100
CrK + DNSCl[c]	39.0 (±1.1)	37.0
Control + mercaptoethanol	105.5 (±1.3)	100
CrK + DNSCl + mercaptoethanol	49.7 (±1.6)	47.1

[a] Buffer is 0.1 M $NaHCO_3$, pH 8.5–9.0. [b] Each value represents the mean numerical average and the maximum deviation of two determinations. [c] Two moles of DNSCl per mole of CrK was added to the reaction mixture.

TABLE IV: Reaction of Creatine Kinase with DNSCl, pH 6.1, and Reactivation with 0.01 M Dithiothreitol.

Sample	Activity, μequiv of ATP/min per mg of Protein	Activity, % of Control
Control	82.5 (±0.5)[a]	100
CrK + DNSCl[b]	3.2 (±4.2)[c]	3.9
Control after column chromatography	94.2 (±9.8)[a]	100
CrK + DNSCl after column chromatography	3.6 (±4.8)[c]	3.8
Control + DTT	83.4 (±2.8)[a]	100
CrK + DNSCl + DTT	61.1 (±8.2)[c]	73.5
Gel-filtered control + DTT	77.8 (±7.7)[a]	100
Gel-filtered, DNS-treated CrK + DTT	52.1 (±3.0)[c]	69.0

[a] Each value represents the mean numerical average and maximum deviation of two determinations. [b] Two moles of DNSCl per mole of CrK was present in the reaction mixture. [c] Each value represents the mean numerical average and maximum deviation of three determinations.

TABLE V: Correlation of Regenerated Activity with Regenerated SH at pH 6.1 after Incubation with 0.01 M Dithiothreitol.

Sample	Activity, μequiv of ATP/min per mg of Protein	Activity, % of Control	Moles of SH/Mole of CrK	% of Control SH's Detected
Control	93.3	100		
CrK + DNSCl[a]	19.5 (±1.5)[b]	29.0		
Control + DTT	104.0	100		
CrK + DNSCl + DTT	81.7 (±2.7)[b]	78.5		
Control + DTT after column chromatography	112.0	100	1.9	100
CrK + DNSCl + DTT after column chromatography	85.9 (±1.5)[b]	76.5	1.6 (±0.03)[b]	84.3

[a] 2.3 moles of DNSCl/mole of CrK was present in the reaction mixture. [b] Each value represents the mean numerical average and maximum deviation of two determinations.

of the sulfenic acid, creatine kinase at 15 mg/ml was treated with a 2 molar excess of DNSCl at pH 6.1 as described above. An aliquot was diluted with glycine and assayed immediately. Another aliquot was incubated with dithiothreitol before assay. The remainder of the reaction mixture was placed over a 1 × 12 cm Sephadex G-25 column in 0.1 M phosphate, pH 6.1, in the cold room. Approximately 1-ml fractions were collected. As soon as the protein came off the column an aliquot was assayed for enzymatic activity. Simultaneously an aliquot was diluted with glycine containing 0.01 M dithiothreitol and allowed to incubate on ice for about 30 min before assay. A control treated with acetone was run under the same conditions.

As seen in Table IV the inactive protein can be reactivated to 70% of the control even after column chromatography. However, ultraviolet spectral examination of the modified protein and examination of the protein-containing tubes with ultraviolet irradiation from a Mineralite lamp revealed the absence of DNS on the protein just as in the case of the enzyme modified with DNSCl in $NaHCO_3$. On the other hand, a strong green fluorescence was observed with the Mineralite lamp in those tubes which contained hydrolysis and/or other breakdown products of DNSCl. When the fluorescent material from the column was pooled and quantitated spectrophotometrically at 312 mμ as described previously, it was found that all the DNSCl put over the column could be accounted for in the fluorescent peak. These data thus indicated that the inactive but reversible intermediate in the reaction of DNSCl with creatine kinase at pH 6.1 was not the thiolsulfonate but, in all probability, the protein sulfenic acid.

The control in this experiment at the concentration of 15 mg/ml had only about 80–86% of the usual activity although the same preparation at 3 mg/ml at pH 6.1 was completely active. Since the isoelectric point of the protein is pH 6.0–6.1 (Noda *et al.*, 1954), it may be that the higher concentration of enzyme leads to aggregation and some precipitation. It has also been found that acetone alone at the concentration used in these experiments will slowly but significantly inhibit creatine kinase. At pH 9.0 and at the time intervals used in the previous experiments, this loss in activity was not appreciable. However, at pH 6.1, near the isoelectric point, and at higher concentrations of protein, the inhibition might be expected to be more severe.

An experiment was designed to prove that regeneration of the activity of DNS-modified protein on incubation with excess thiols was due to the actual restoration of free sulfhydryls by disulfide exchange

$$\text{Protein–SOH} + \text{RSH} \longrightarrow \text{Protein–S–SR} + H_2O \quad (5)$$

$$\text{Protein–S–SR} + \text{RSH} \longrightarrow \text{Protein–SH} + \text{RSSR} \quad (6)$$

Creatine kinase (11.3 mg/ml) was treated with 2.3 molar excess of DNSCl on 0.1 M NaH_2PO_4, pH 6.1, for 5 min at 4°. An aliquot was removed for assay. The remaining reaction mixture was incubated in 0.01 M dithiothreitol for 50 min at 4°. After removal of an aliquot of the incubation mixture for enzymatic assay, the remainder was placed over Sephadex G-25 in 0.05 M NaH_2PO_4 to remove the excess dithiothreitol. A control was run under the same conditions. After gel filtration the enzyme was reassayed and an aliquot was reacted with DTNB to quantitate the amount of free sulfhydryls in the control and DNS-treated protein.

The results in Table V showed that the activity of creatine kinase could be regenerated from about 21 to 79% of the control activity with dithiothreitol. Furthermore, the regeneration of activity to 79% of the control value could be correlated with restoration of the reactive sulfhydryls in the enzyme to 85% of the control –SH's. These data support the previous observations that loss of activity of creatine kinase on reaction with DNSCl is associated with loss of free sulfhydryl groups.

The above results suggest that at pH 6.1 creatine kinase is oxidized to protein–SOH on treatment with DNSCl. At higher pH values oxidation apparently proceeds to the level of the sulfinic and/or sulfonic acids which cannot then be reduced under the mild conditions used in these experiments.

Discussion

The present study provides the first evidence for the chemical reaction of the fluorescent chromophore DNSCl with a sulfhydryl group in proteins. The sulfhydryls in creatine kinase are sterically restricted and thus do not appear to be oxidized to the disulfide (Rosenberg and Ennor, 1955;

Trundle and Cunningham, 1969). This fact and the consumption of 2, rather than only 1, moles of DNSCl at pH 6.1 diminish the possibility that the disappearance of SH is caused by formation of an intramolecular disulfide linkage. Since the intermolecular linkage can be eliminated (see below), the reaction presumably occurs by the formation and breakdown of a thiolsulfonate intermediate followed at higher pH values by oxidation of the PSOH intermediate, according to eq 1, 3, and 4. The steric restriction of the sulfhydryls in this enzyme thus allows investigation of the oxidation of thiols in aqueous media without disulfide formation.

Creatine kinase contains a total of eight half-cystine residues (Yue *et al.*, 1967; Bayley and Thomson, 1967). DNSCl reacts with only the two "active site" sulfhydryls in the native enzyme to produce an inactive enzyme at pH 6.1 and 4° and at pH 8.5–9.0 at room temperature. The inactivation of the enzyme at pH 6.1 is reversible with dithiothreitol. This might be interpreted as formation of a disulfide, the presence of a thiolsulfonate intermediate, or oxidation to the sulfenate level. If disulfide formation had occurred either the stoichiometry of the reaction should have been close to 1 mole or DNSCl instead of 2 (see eq 1 and 2) or 4 sulfhydryl groups should have disappeared. Furthermore, the stoichiometry of iodine oxidation indicated that none of the six sulfhydryls in creatine kinase was in a position sterically favorable for disulfide formation, and ultracentrifugation of the iodine-oxidized protein revealed that intermolecular disulfide formation did not occur under these conditions (Trundle and Cunningham, 1969). The thiolsulfonate intermediate was ruled out by the fact that DNS-treated creatine kinase after Sephadex G-25 filtration, when examined spectrophotometrically and with an ultraviolet Mineralite lamp, disclosed no DNS attached to the protein. This gel-filtered protein, although inactive when it came off the column, could be reactivated with dithiothreitol. All of the DNSCl in the reaction mixture which was put over the column could be accounted for in the peak containing hydrolytic products of DNSCl. In addition, regeneration of enzyme activity with dithiothreitol was attributed to restoration of the "active site" sulfhydryls as illustrated by eq 5 and 6. The existence of a protein–SOH intermediate consequently is offered as the most likely explanation for these results. The reversal of the inhibition of activity suggests that the sulfhydryls can be partially oxidized without permanently destroying the three-dimensional structure of the active site.

Although sulfenic acid intermediates are usually considered unstable in organic reactions (Savige and MacLaren, 1966), the environment of the protein–SOH in creatine kinase at pH 6.1 and 4° apparently stabilizes this intermediate. Similar results were found on iodine oxidation of this protein (Trundle and Cunningham, 1969). In addition, the stoichiometry of the iodine oxidation of the sulfhydryls in bovine serum albumin (Hughes and Straessle, 1950), triosephosphate dehydrogenase (Ehring and Colowick, 1964), TMV (Fraenkel-Conrat, 1955), and lactoglobulin and ovalbumin (Cunningham and Nuenke, 1959) indicates oxidation states beyond the disulfide. In all these cases the oxidation is easily reversible with excess thiols and the oxidation product of TMV, lactoglobulin, and ovalbumin has been identified as the sulfenyl iodide. Oxidation to the sulfenic level has also been suggested in the reaction of a catalytically active SH of glyceraldehyde 3-phosphate dehydrogenase with *o*-iodoso benzoate (Parker and Allison, 1969).

Inactivation at pH 8.5–9.0 also has been attributed to the oxidation of the two active site sulfhydryls. However, the stoichiometry observed in this case was close to 4 moles of DNSCl per mole of creatine kinase instead of 2 moles of reagent per mole of enzyme. This phenomenon was apparently a result of the increased rate of reaction of DNSCl with the enzyme at pH 6.1 or to an increased spontaneous hydrolysis of DNSCl in the bicarbonate buffer at the higher pH and higher temperature.

The mechanism by which oxidation beyond the proposed sulfenate to sulfinic and/or sulfonic acids occurs at pH values near 9.0 is not known. The pH dependence itself might indicate oxidation *via* an ionic rather than a free-radical mechanism. The effect of pH on oxidations of thiols is incompletely understood and further complicated by the fact that the rates of oxidation vary in different buffers at the same pH (Cecil and McPhee, 1959). Cysteine and reduced glutathione show optimal rates of oxidation at alkaline pH and Cecil and McPhee suggest the involvement of a mercaptide rather than the undissociated sulfhydryl.

The results of this study indicate that caution should be used in evaluating the effects of the introduction of DNS groups into proteins which contain sulfhydryl groups and particularly in evaluating fluorescence and fluorescence depolarization studies of such modified proteins. The possible modifications of the sulfhydryl groups could create changes in protein conformation and reactivity which might be mistakenly attributed to the DNS group(s) bound at other sites. The preliminary reversible blockage of the sulfhydryl groups with tetrathionate (Liu, 1967; Kassab *et al.*, 1968) or dinitrofluorobenzene (Shaltiel, 1967) prior to reaction with DNSCl provides adequate means to bypass this difficulty in most cases.

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