The Role of Sulfhydryl Groups in the Catalytic Function of Isocitrate Dehydrogenase

III. EFFECT OF N-ETHYLMALEIMIDE ON CHEMICAL AND PHYSICAL PROPERTIES*

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SUMMARY

Incubation of the TPN-dependent isocitrate dehydrogenase from pig heart with 1-14C-N-ethylmaleimide (NEM) leads to inactivation and altered Michaelis constants for isocitrate and oxalosuccinate concomitant with incorporation of 2 moles of reagent. Reaction of enzyme with radioactive NEM in the presence of isocitrate and Mn2+ yields a catalytically functional enzyme, with the same altered Michaelis constants, which also contains 2 moles of reagent. Inactive enzyme with a single carboxymethyl methionyl residue exhibits the same ability to bind radioactive NEM as does native enzyme; however, prior inactivation by reaction of 5 sulfhydryl groups with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) prevents binding of NEM.

Cysteine is the sole type of amino acid modified in the inactive NEM-enzyme, as determined by paper chromatographic identification of radioactive cysteinosuccinic acid and S-(N-ethylsuccinimido)cysteine in acid and proteolytic digests of 14C-labeled modified enzyme. Thirteen moles of cysteic acid are found after performic acid oxidation of native enzyme, the same as the number of free -SH groups observed by reaction with DTNB after denaturation; therefore, no disulfide bonds are present in isocitrate dehydrogenase. A decrease in the number of measurable -SH groups after reaction with NEM is consistent with modification of cysteinyl residues. By contrast, the recovery of NH-terminal alanine after the Edman reaction was approximately the same for native and modified enzyme.

Inactive enzymes with a single carboxymethyl methionyl residue, with 2 or 5 altered sulfhydryl groups, give a reaction of antigenic identity to that of native enzyme as measured against rabbit antibody to isocitrate dehydrogenase. The molecular size of the inactive NEM-enzyme is the same as that of native enzyme, as determined by gel filtration on Sephadex G-150, and the rates of protolytic digestion by Pronase are not significantly different. These data argue against a generalized structural change in the modified enzyme. However, a small conformational change is indicated by a decrease in the reaction rate of DTNB with the residual -SH groups of the inactive NEM-enzyme. The magnitude of conformational change is not directly related to loss in enzymatic activity, since an observed decrease in the amplitude of the optical rotatory dispersion curves is greater for active NEM-enzyme ([α]234 -5610°) than it is for inactive NEM-enzyme ([α]234 -7430°), as compared to native isocitrate dehydrogenase ([α]234 -8050°). These results, in conjunction with those of the preceding paper, suggest that, in the absence of isocitrate and MnSO4, modification of Sulfhydryl Group A produces inactivation, whereas modification of Sulfhydryl Group B leads to a small conformational change which is reflected in altered Michaelis constants. In the presence of substrates, NEM reacts with 2 sulfhydryl groups, including Group B but not Group A, which produces a further change in the conformation of the enzyme but no concomitant change in the kinetic properties.

This paper constitutes part of a continuing effort to identify and elucidate the function of the amino acid residues in the active site of isocitrate dehydrogenase (threo-2,4-dihydroxyacetone phosphate dehydrogenase, EC 1.1.1.42). Evidence has been presented that a single methionyl residue is essential for catalysis (1, 2). This enzyme was the first dehydrogenase for which a critical role for methionine was indicated; however, methionyl residues have recently been implicated also in the thiolase malarial dehydrogenase from pig heart (3). Analyses of pH-rate profiles of isocitrate dehydrogenase in water and 20% ethanol suggested the requirement for activity of the dissociated form of an enzyme carboxyl group (2). An additional involvement of sulfhydryl groups was demonstrated by the inactivation produced by reaction of 5 cysteinyl residues with 5,5'-dithiobis(2-nitrobenzoic acid) (4).

In the preceding paper (5) it is shown that incubation of isocitrate dehydrogenase with N-ethylmaleimide results in a loss of both the dehydrogenase and decarboxylase functions of the enzyme. Furthermore, partially active enzymes exhibit altered Michaelis constants for isocitrate and oxalosuccinate, as well as an increase in the value of pK for the active enzyme-substrate complex. NEM has been shown to react with sulfhydryl and

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1 The abbreviations used are: NEM, N-ethylmaleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PTH, phenylthiohydantoin.
α-amino groups in amino acids and proteins (6-8). This paper focuses on the number and nature of the amino acid residues of isocitrate dehydrogenase modified by NEM and responsible for the changes in the kinetic properties described in the preceding paper (5). In addition, it considers the effect of modification on the tertiary structure of the protein.

**Experimental Procedure**

**Materials**—Pig heart TPN-dependent isocitrate dehydrogenase was supplied as a solution in 50% glycerol by Boehringer-Mannheim Corporation and was further purified to homogeneity (2). Carboxymethyl-isocitrate dehydrogenase, specifically modified at a single methionyl residue, was prepared by incubation of 2.0 mg per ml of enzyme with 2.8 \( \times 10^{-3} \) M iodoacetate at pH 5.8 and 30° (2). DTNB-enzyme, with 5 modified sulfhydryl groups, was prepared by incubation of isocitrate dehydrogenase (1.1 mg per ml) at 25° with 2.54 \( \times 10^{-4} \) M 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M triethanolamine chloride buffer, pH 7.7, containing 0.3 M NaSO4, 1 mM EDTA, and 10% glycerol (4).

Substrates and coenzymes were obtained from the same companies and standard assays were conducted as specified in the preceding paper (5). Aldrich Chemical Company supplied the 5,5'-dithiobis(2-nitrobenzoic acid).

\( \text{N-Ethylmaleimide-}^{14} \text{C} \) was purchased from Schwarz Bio-Research, Inc. The concentration of NEM was determined spectrophotometrically by its absorbance at 302 m\( \mu \) (9). A measured amount of NEM was converted to a nonvolatile derivative with excess cysteine for the measurement of its specific radioactivity by means of a Nuclear-Chicago gas flow counter. Evidence for its radiopurity was obtained by reaction of NEM-\( \cdot \)\( ^{14} \text{C} \) with either radioactive or unlabeled o-mercaptobenzoic acid.

\( \text{S-(N-Ethylsuccinimido)cysteine} \) was prepared by incubation of cysteine and \( \text{N-ethylmaleimide-}^{14} \text{C} \) at pH 5.0 and 30° for 30 hours. The cyclic adduct was subjected to the same conditions as was the radioactive NEM-enzyme.

**Digestion of NEM-\( \cdot \)\( ^{14} \text{C} \)-enzyme**—Radioactive NEM-enzyme of 2 to 4% residual activity was prepared by reaction of native enzyme with 4.4 \( \times 10^{-4} \) M \( \text{N-ethylmaleimide-}^{14} \text{C} \) at pH 7.7 and 30° for 60 min in 0.08 M triethanolamine chloride buffer, pH 7.7, containing 0.24 M NaSO4 and 8% glycerol. Excess reagent was removed by exhaustive dialysis at 4° against 0.1 M triethanolamine chloride buffer, pH 7.7, containing 0.3 M sodium sulfate and 10% glycerol, prior to proteolytic digestion. (Unless otherwise specified, these are the conditions used throughout this paper to prepare NEM-enzyme, either radioactive or unlabeled, with 2 modified sulfhydryl groups.) Prior to acid hydrolysis, the enzyme was dialyzed against distilled water.

Modified enzyme was incubated with Pronase (10:1) for 48 hours at pH 7.7 and 40°, followed by treatment with carboxypeptidase A and B (each present at a weight ratio of 20:1) for 18 hours at 40°. Hydrolysis was approximately 93% complete as monitored by the quantitative reaction of the digest with ninhydrin, using leucine as a standard (11). Digests were desalted on Dowex 1 and Dowex 50, and paper chromatography was conducted as described under "Results." Acid hydrolysis was conducted in 6 N HCl in evacuated sealed tubes at 110° for 24 to 72 hours.

**Quantification of Amino-terminal Group**—The Edman degradation was conducted by the paper strip method of Fraenkel-Conrat, Harris, and Levy (12). Native and NEM-enzyme (4.5 to 5.0 mg) were dialyzed against distilled water before being transferred to four paper strips (1 × 3 cm). The \( \text{I}^{14} \text{N} \) derivative was identified as PTI-alanine by descending chromatography on Whatman No. 1 filter paper in Solvent I (\( R_\text{F} \) 0.16) and Solvent II (\( R_\text{F} \) 0.37) of Sjoquist (13).

**Quantification of Cysteic Acid**—The cysteic acid content of isocitrate dehydrogenase, dialyzed against distilled water, was determined by performic acid oxidation (14) followed by hydrolysis in 6 N HCl at 110° for 20 hours in sealed tubes flushed with nitrogen and evacuated. Comparison of chromatograms of digests of oxidized enzyme from the amino acid analyzer with the previously determined amino acid composition of isocitrate dehydrogenase (1) allowed determination of the number of moles of cysteic acid per mole of enzyme. Glutathione, subjected to the same treatment, was used as a standard to correct for the recovery of cysteic acid.

**Preparation of Antisera to Isocitrate Dehydrogenase**—Rabbit antisera were obtained by subcutaneous injection of each of three animals with 0.5 mg of native isocitrate dehydrogenase emulsified with Freund's adjuvant. Two injections were given 3 weeks apart, and the rabbits were bleed every 2 to 3 weeks starting 1 month after initiation of the immunization schedule. Another group of three rabbits was immunized with 0.5 mg each of carboxymethyl enzyme, containing a single modified methionyl residue, which had been freed of excess iodoacetate. Control sera were collected prior to injection of the animals. Antisera were examined by Ouchterlony double diffusion in agar plates. For some experiments, the γ-globulin fraction was purified by passage over a column of DEAE-cellulose equilibrated with 0.01 M potassium phosphate buffer, pH 7.6, containing 0.015 M NaCl. Anti-isocitrate dehydrogenase emerged at the solvent front.

**Optical Rotatory Dispersion**—Optical rotatory dispersion spectra were obtained for native and modified enzymes with a Cary model 60 spectropolarimeter in the wave length region from 320 to 220 m\( \mu \). Experiments were performed at 23° in 0.12 M triethanolamine chloride buffer, pH 7.4, containing 0.03 M NaSO4 and 1% glycerol.

**Results**

**Binding of NEM-\( \cdot \)\( ^{14} \text{C} \) by Enzyme**

**Native Enzyme**—Incubation of pig heart TPN-dependent isocitrate dehydrogenase with \( \text{N-ethylmaleimide-}^{14} \text{C} \) at pH 7.7 and 30° leads to incorporation of radioactivity concomitant with inactivation, as shown in Table I, and altered kinetic parameters including an increase in the Michaelis constant for isocitrate (5). The excess reagent was removed either by gel filtration (Method I) or by dialysis (Method II); by both methods, however, approximately 2 moles of NEM were bound per mole of inactive enzyme. A maximum of 2 amino acid residues are implicated in the catalytic activity of the enzyme and in the binding of isocitrate and oxisalicycinate.

Reaction with NEM-\( \cdot \)\( ^{14} \text{C} \) in the presence of isocitrate and
MnSO₄ produces a fully active enzyme but one with altered kinetic parameters (2). Approximately 2 moles of NEM are incorporated per mole of protein. It is apparent that substrates must alter the specificity of NEM for sites on the enzyme, but not the total number of groups incorporated. It is likely that one of the modified amino acid residues is the same in both enzyme preparations, since the alterations in kinetic parameters are similar, but that the second residue is distinct, since one preparation is fully active, as measured at high substrate concentrations, and the other is inactive.

\[ \text{DTNB and Carboxymethyl Enzyme—Table I shows that prior reaction of 5 sulfhydryl groups with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB-enzyme) almost totally prevents subsequent reaction with radioactive NEM. The initial treatment with DTNB yielded a preparation with 10\% residual activity, and thus the measured incorporation of 0.35 moles of NEM per mole of protein can largely be accounted for by binding of 2 moles per mole protein by the residual active enzyme. These results indicate that the 2 cysteinyl residues attacked by NEM in the native enzyme are among the 5 -SH groups modified by DTNB.} \]

It has previously been shown that carboxymethylation of a single essential methionyl residue of isocitrate dehydrogenase blocks disulfide exchange with DTNB of 1 of the reactive sulfhydryl groups (4). Proximity between a sulfhydryl and a methionyl residue in the active site was therefore suggested. In contrast, the results of Table I indicate that, under the standard conditions of incubation, the carboxymethyl enzyme is fully competent to bind the smaller sulfhydryl reagent, N-ethylmaleimide. Since the essential sulfhydryl groups attacked by DTNB and by NEM appear to be identical, the different effect of prior blockage of the methionyl residue on reaction with these two compounds yields an estimate of the distance between the critical sulfhydryl and methionyl residues.

\[ \text{Identification of Amino Acid Residues Modified by NEM} \]

\[ \text{Paper Chromatography of Digests of } { }^{14} \text{C-NEM-enzyme—N-} \]

Ethylmaleimide is usually assumed to react with sulfhydryl groups in proteins, although ample evidence exists for reaction with the \( \alpha \)-amino group of amino acids and proteins (6-8). Consequently, efforts were made to identify directly the types of amino acid residues modified by NEM in isocitrate dehydrogenase. Inactive NEM-enzyme was digested with 6 \( \times \) HCl for 24 to 72 hours along with an authentic sample of \( S-(N\text{-ethylsuccinimidol}-\)cysteine and then subjected to paper chromatography in two different solvent systems. Fig. 1 reproduces the radioactive peaks for the 24-hour hydrolysate as located by a paper strip scanner on a chromatogram run with 1-butanol-pyridine-acetic acid-water as solvent. The NEM-enzyme preparation was used.

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the terminal amino acid had reacted with N-ethylmaleimide, both enzyme. The residual free sulfhydryl content of the inactive protein, which is consistent with a modification of a small number of cysteinyl residues by NEM.

Each case, with an enzyme as shown in Table III is 11.8 moles per mole of cysteic acid, as measured after performic acid oxidation. The enzyme does not appear to contain disulfide bonds, since the acid hydrolysis, is also approximately 13 moles per mole of cysteic acid content, as measured after performic acid oxidation.

After desalting, this digest was examined by paper chromatography, as above. Only one radioactive peak was observed in each case, with an RF characteristic of the initially formed cyclic adduct of NEM and cysteine. Alternatively, the radioactive NEM-enzyme was subjected to enzymatic digestion by Pronase, followed by carboxypeptidases A and B, as described under “Experimental Procedure.” After desalting, this digest was examined by paper chromatography, as above. Only one radioactive peak was observed in each case, with an RF characteristic of the initially formed cyclic adduct of NEM and cysteine, S-(N-ethylsuccinimido)cysteine acid, in which 90% of the radioactivity appeared at the RF value characteristic of S-cysteinosuccinic acid.

Alternatively, the radioactive NEM-enzyme was subjected to enzymatic digestion by Pronase, followed by carboxypeptidases A and B, as described under “Experimental Procedure.” After desalting, this digest was examined by paper chromatography, as above. Only one radioactive peak was observed in each case, with an RF characteristic of the initially formed cyclic adduct of NEM and cysteine, S-(N-ethylsuccinimido)cysteine, as shown in Table II. It thus appears that all of the radioactivity found in the digests can be accounted for as derivatives of NEM and cysteine.

**Chemical and Physical Characteristics of NEM and Native Enzyme**—Thirteen free sulfhydryl groups are present in the native enzyme, as determined by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) under denaturing conditions (Table III). The enzyme does not appear to contain disulfide bonds, since the cysteic acid content, as measured after performic acid oxidation and acid hydrolysis, is also approximately 13 moles per mole of enzyme. The residual free sulfhydryl content of the inactive NEM enzyme as shown in Table III is 11.8 moles per mole of protein, which is consistent with a modification of a small number of cysteinyl residues by NEM.

In order to ascertain whether the amino group of the NH-terminal amino acid had reacted with N-ethylmaleimide, both the native and inactive NEM-enzyme were subjected to reaction with phenylisothiocyanate, as described under “Experimental Procedure.” Table III records a recovery of 0.76 mole of PTH-amino acid, values which are comparable within the experimental error of this method. Since a blocked NH-terminal would have been indicated by a decreased yield of PTH-amino acid, it appears that there was no significant reaction of NEM with the NH-terminal amino acid. The incorporation of 2 moles of reagent can thus be attributed only to reaction at cysteine residues.
The molecular size of isocitrate dehydrogenase is not altered as a result of reaction with N-ethylmaleimide, as determined by gel filtration on Sephadex G-150 under the conditions given in Table III. The Stokes radius of native enzyme was determined to be 3.90 nm by the procedure of Ackers (17) using cytochrome c, chymotrypsin, bovine serum albumin, lactate dehydrogenase, and dextran blue for column calibration. NEM-enzyme of 31% residual activity when applied to the same column exhibited a single protein peak without appreciable broadening at 63.6 ml, as compared to 63.7 ml for the native enzyme.

Combination with Antibody

The ability of native and modified isocitrate dehydrogenase to combine with rabbit antibody to native enzyme was tested by means of the Ouchterlony double diffusion technique. Enzyme treated with 5,5'-dithiobis(2-nitrobenzoic acid) gives a reaction of antigenic identity to that of native enzyme with the antiserum (Fig. 2A). In this enzyme, 5 sulfhydryl groups have been altered, although only 2 of these residues at most are directly involved in catalysis (4). Control sera, obtained from animals prior to injection, gave no precipitin band. Inactive NEM-enzyme with 2 altered sulfhydryl groups exhibits reaction of identity to that of the native enzyme with antibody (Fig. 2B). The indication is that these modification reactions have produced relatively specific changes, rather than a generalized conformational change, and that it has not noticeably interfered with the ability of isocitrate dehydrogenase to combine with antibody to the native enzyme.

Inactive carboxymethyl enzyme, with a single modified methionyl residue (2), also gave a reaction of identity to that of native enzyme with antisera (Fig. 3). Similarly, both enzymes gave identical reactions when tested against antisera prepared by injection of rabbits with the carboxymethyl enzyme. This specific modification of the catalytic site also does not interfere significantly with combination of the enzyme with antibody.

In order to verify that the precipitation observed represented reaction with the enzyme, γ-globulin purified from antisera was incubated with native enzyme for 2 hours at 37°C followed by 15 hours at 4°C. After centrifugation of the resulting precipitate, the supernatant exhibited only 43% of its original activity. Enzyme incubated under the same conditions with γ-globulin purified from animals prior to injection yielded no precipitate and no decrease in enzymatic activity. Loss of activity upon precipitation does not demonstrate combination of antibody at the active site; it merely indicates that the enzyme is inactive in the aggregated state. In order to test for the presence of active but soluble complexes of enzyme and antibody, the supernatant from the experiment was subjected to gel filtration on a
FIG. 3. Immunological comparison of native and carboxymethyl isocitrate dehydrogenase by Ouchterlony double diffusion in 1% agar. The center well contained antisera produced by injection of native enzyme into rabbits, as described under “Experimental Procedure.” The outer wells contained: A, C, and E, different preparations of carboxymethyl enzyme at concentrations of 0.27, 0.21, and 0.21 mg per ml, respectively; B, D, and F, native enzyme at concentrations of 0.20, 0.60, and 0.40 mg per ml, respectively. The photograph was taken approximately 18 hours after the samples were applied.

column of Sephadex G-150 (2.3 × 29.5 cm). On this column, dextran blue elutes with a peak at Fraction 26 (1.48 ml per fraction), γ-globulin elutes at Fraction 31, and native isocitrate dehydrogenase elutes at Fraction 40. Combination of functional enzyme with antibody would be indicated by an increase in the size of the enzymatically detectable species, as reflected by elution in an earlier fraction than γ-globulin. When the γ-globulin-isocitrate dehydrogenase supernatant was applied to the column, 95% of the enzymatic activity emerged with a peak at Tube 40, demonstrating that most of the residual activity represented excess, uncombined enzyme. However, 5% of the activity was observed with a peak at Fraction 27, suggesting the possibility of combination of the enzyme and antibody to produce a catalytically functional complex. These results contrast with those for some other enzymes, a well documented example of which is the inhibition of Staphylococcus nuclease in soluble complexes of nuclease-antinuclease (18).

Another approach taken to determine whether the major sites for reaction of antibody with enzyme include the active site was to conduct the double diffusion experiments in agar plates containing substrates. No difference in the precipitin lines formed between native enzyme and antibody was observed in plates containing 2 mM MnSO₄ alone or 2 mM MnSO₄ combined with 4 mM isocitrate, 0.2 mM TPNH, 0.1 mM TPN, or 10.0 mM α-ketoglutarate. These concentrations of substrates are at least 20 times the kinetically determined Michaelis constants. The major locus of combination of enzyme and antibody is probably not at the catalytic substrate-binding sites, since there is no visible effect of substrates on the precipitin reaction. Any conformational changes effected by the substrates do not appear to interfere significantly with combination of enzyme and antibody.

**Optical Rotatory Dispersion**

Fig. 4 shows the optical rotatory dispersion curve of native isocitrate dehydrogenase along with that of both inactive and active NEM-enzymes, prepared respectively by incubation with reagent in the absence and presence of isocitrate and MnSO₄. A small decrease is observed in the specific rotation of the inactive preparation (Curve B) in the region from 260 to 225 μm. The specific rotation at 234 μm is -8050° for the native enzyme and -7430° for the inactive preparation. This change has been confirmed by monitoring the decrease in specific rotation at 234 μm as a function of time of incubation of enzyme with 2.24 × 10⁻⁷ M or 4.48 × 10⁻⁷ M N-ethylmaleimide. However, the magnitude of the change is minor as compared to that observed for the 100% active NEM-enzyme (Curve C); in this case the specific rotation at 234 μm is -5610°. It is apparent that the extent of conformational alteration upon exposure to NEM is not necessarily related to loss of activity.
Role of Sulfhydryl Groups in Isocitrate Dehydrogenase. III

As described in the preceding paper (5), NEM causes both inactivation and an alteration in the affinity for oxaloacetate and isocitrate, as well as an increase in the $pK_a$ for the enzyme-substrate complex ($pK_{as}$). All of these effects on NEM can now be attributed to reaction at 2 sulfhydryl groups, A and B, respectively. Incubation of the enzyme with NEM in the presence of isocitrate and MnsO$_4$ produces a fully active preparation, as measured at high substrate concentrations, but one which still exhibits the same abnormal Michaelis constants and $pK_{as}$ values. Again, 2 moles of $^{14}$C-labeled NEM are incorporated. The implication is that NEM reacts with 1 sulfhydryl group that is the same in the presence and absence of substrates. It is the modification of this sulfhydryl (Group B) which causes alterations in the kinetic parameters of the enzyme (5). The additional sulfhydryl group which reacts with NEM may be different in the absence (Group A) and presence (Group C) of substrate, since one preparation is catalytically competent, whereas the other is inactive. A single sulfhydryl group (Group A) thus seems to be implicated directly in the catalytic process.

Other examples exist in which the identical number of amino acid residues are modified under two sets of conditions, although the specific groups involved appear to be different. The same number of amino acid groups are acetylated when glutamate dehydrogenase is treated with acetic anhydride in the presence of ADP as when the reaction is conducted with added GTP; however, one preparation is enzymatically functional while the other is inactive (19). The specificity of a reagent for amino acid residues of a protein can thus be changed by the presence of substrates or modifiers of an enzyme, and therefore a strict reliance on the number of altered residues can be misleading. It is also possible that incorporation of 1 mole of reagent per mole of protein can represent partial reaction at several groups, as in the case of tryptophan synthetase (20). Isolation of the particular peptides of isocitrate dehydrogenase which are labeled by NEM in the presence and absence of substrates is currently in progress in this laboratory in order to elucidate these proteins.

The inactivation of isocitrate dehydrogenase by 5,5'-dithiobis-(2-nitrobenzoic acid) (4) showed that 5 sulfhydryl groups were altered in the inactive enzyme. Only 3 —SH groups reacted in the presence of isocitrate and MnsO$_4$, producing an active enzyme. A maximum of 2 —SH groups were thereby implicated in the loss of activity, providing the assumption was made that the presence of substrates did not lead to reaction of DTNB with a different set of —SH groups. The present results with NEM suggest that the 2 groups modified by this reagent are among the 5 which react with DTNB (Table I) and that modification of these residues is sufficient to inactivate the enzyme.

Carboxymethylation of a single essential methionyl residue with iodoacetate (2) blocks 1 of the sulfhydryl groups normally protected by isocitrate and manganous sulfate against DTNB (4). These data were interpreted to indicate a proximity between a sulfhydryl and a methionyl residue in the active site. It is apparent from Table I, however, that carboxymethylation of the methionyl residue does not interfere with reaction of NEM at 2 —SH groups. Similarly, modification of methionine does not significantly impair the ability of the inactive enzyme to bind radioactive isocitrate and $\alpha$-ketoglutarate (21), at least the first of which must bind in the vicinity of the critical sulfhydryl group. It appears likely that the extent of influence exerted by carboxymethyl methionine on binding at an adjacent site will depend on the size of the ligand. In the case of a small

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**DISCUSSION**

In seeking to interpret the effects of chemical modification of an enzyme, it is important to ascertain that reaction takes place at specific and limited sites on the protein. The results reported in this paper indicate that 2 moles of reagent are bound by the enzyme under denaturing conditions (Table III), only 5.17 —SH groups react under the milder conditions given in the Fig. 5 legend and described previously (4). Under the same conditions, the inactive NEM-enzyme, containing 2 blocked sulfhydryl groups, exhibits further reaction of 5.95 sulfhydryl groups with DTNB, as determined spectrophotometrically at 412 nm by the method of Ellman (16). Little differentiation is observed in the reactivity of the 5 to 6 —SH groups of each enzyme, since the reaction follows the pseudo first order kinetics, with a single characteristic rate constant. However, there is a striking difference in the rates of reaction of the native and NEM-enzyme with this reagent, as shown in Fig. 5; the rate constant for the NEM-enzyme is only 4% of that for the native enzyme.

**Reactivity of Free Sulfhydryl Groups**

Although all 13 sulfhydryl groups of unmodified isocitrate dehydrogenase react with 5,5'-dithiobis(2-nitrobenzoic acid) under denaturing conditions (Table III), only 5.17 —SH groups react under the milder conditions given in the Fig. 5 legend and described previously (4). Under the same conditions, the inactive NEM-enzyme, containing 2 blocked sulfhydryl groups, exhibits further reaction of 5.95 sulfhydryl groups with DTNB, as determined spectrophotometrically at 412 nm by the method of Ellman (16). Little differentiation is observed in the reactivity of the 5 to 6 —SH groups of each enzyme, since the reaction conforms to pseudo first order kinetics, with a single characteristic rate constant. However, there is a striking difference in the rates of reaction of the native and NEM-enzyme with this reagent, as shown in Fig. 5; the rate constant for the NEM-enzyme is only 4% of that for the native enzyme.

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In seeking to interpret the effects of chemical modification of an enzyme, it is important to ascertain that reaction takes place at specific and limited sites on the protein. The results reported in this paper indicate that 2 moles of reagent are bound by the enzyme under denaturing conditions (Table III), only 5.17 —SH groups react under the milder conditions given in the Fig. 5 legend and described previously (4). Under the same conditions, the inactive NEM-enzyme, containing 2 blocked sulfhydryl groups, exhibits further reaction of 5.95 sulfhydryl groups with DTNB, as determined spectrophotometrically at 412 nm by the method of Ellman (16). Little differentiation is observed in the reactivity of the 5 to 6 —SH groups of each enzyme, since the reaction conforms to pseudo first order kinetics, with a single characteristic rate constant. However, there is a striking difference in the rates of reaction of the native and NEM-enzyme with this reagent, as shown in Fig. 5; the rate constant for the NEM-enzyme is only 4% of that for the native enzyme.
molecule, such as NEM or isocitrate, the influence is minimal; however, the binding of a large compound such as 5,5'-dithiobis-(2-nitrobenzoic acid) is significantly inhibited by modification of the methionyl residue.

In analyzing the effects of chemical modification on a protein it is necessary to consider the possibility of changes in its structure as well as its function. A generalized conformational change in the inactive NEM-enzyme is unlikely on the basis of the antibody-binding results, the similar rates of proteolysis of the native and modified enzymes, and the identical molecular size. However, some change in the conformation of this modified enzyme is suggested by the decrease in the reaction rate of the residual sulfhydryl groups with 5,5'-dithiobis(2-nitrobenzoic acid) (Fig. 5). These results contrast with the previously reported effect of the presence of substrates on reaction of native acid) (Fig. 5). These results contrast with the previously reported effect of the presence of substrates on reaction of native enzyme. An analogous decrease in reactivity toward DTNB of residual—SH groups has been observed after carboxymethyl-ation of the active site cysteine residues of lobster glyceraldehyde 3-phosphate dehydrogenase (22).

The relative reactivity of the sulfhydryl groups of isocitrate dehydrogenase toward DTNB and NEM is of interest. It was pointed out previously (3) that the 5—SH groups which readily undergo a disulfide exchange reaction with DTNB do not differ markedly in their reactivity, despite the fact that a single residue is presumably involved directly in catalysis and this residue might have been expected to be unusually reactive (23, 24). By contrast, only 2 of these —SH groups exert to any significant extent with NEM. There are many examples of cysteine residues of proteins which exhibit differential reactivity toward compounds which undergo mechanistically distinct reactions (24–26) or toward reagents participating in similar reactions but which are electrostatically or sterically distinct (20, 27). The critical sulfhydryl groups of isocitrate dehydrogenase are distinguished in reactivity in an addition reaction, but are unremarkable in a disulfide exchange reaction. This distinction may provide a clue as to the role of the sulfhydryl group in the catalytic function of this enzyme.

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