The Role of Sulfhydryl Groups in the Catalytic Function of Isocitrate Dehydrogenase. I. Reaction with 5,5′-Dithiobis(2-nitrobenzoic acid)*

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ABSTRACT: The dehydrogenase and decarboxylase activities of the triphosphopyridine nucleotide dependent isocitrate dehydrogenase are both lost as a result of reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Protection against inactivation is provided by isocitrate plus manganous sulfate and by reduced triphosphopyridine nucleotide plus manganous sulfate, but not by isocitrate, reduced pyridine nucleotide, or metal ion separately. A kinetic treatment is presented describing the initial velocity of an enzymatic reaction in the presence of a reversible inhibitor which reacts slowly but which competes with the substrate for the same or mutually exclusive sites. The rate of inactivation of isocitrate dehydrogenase by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of varying concentrations of isocitrate or reduced triphosphopyridine nucleotide and manganous sulfate is analyzed as a special case of this general mechanism. Alteration of the kinetic parameters of the enzyme is not responsible for the observed loss of activity of isocitrate dehydrogenase, since partially active preparations exhibit Michaelis constants and pH–rate profiles which are essentially identical with those of native enzyme. Incubation of the modified enzyme with mercaptoethanol results in a slow reactivation. Determination of the number of reactive sulfhydryl groups by the increase in absorbance at 412 mμ accompanying the release of 2-nitro-5-mercaptobenzoate from the reagent or by the enhanced absorbance at 323 mμ of the isolated modified enzyme yields a value of approximately five altered residues in the inactive enzyme. Only three SH groups react in the presence of isocitrate or reduced triphosphopyridine nucleotide and $MnSO_4$, producing an active enzyme; a maximum of two sulfhydryl groups is thereby implicated in the loss of activity. Carboxymethylation of a single essential methionyl residue (Colman, R. F. (1968), *J. Biol. Chem. 243*, 2454) blocks one of the SH groups normally protected by isocitrate and manganous ion, demonstrating proximity between a sulfhydryl and a methionyl residue in the active site.

Isocitrate dehydrogenase (*threo*-D_S-isocitrate:NADP oxidoreductase (decarboxylating), EC 1.1.1.42) catalyzes two mechanistically distinct reactions: a pyridine nucleotide dependent oxidation yielding oxalosuccinate, followed by decarboxylation of the β-keto acid to produce α-ketoglutarate (Ochoa, 1948; Moyle and Dixon, 1955; Siebert *et al.*, 1957a). The over-all reaction appears to proceed through an enzyme-bound oxalosuccinate intermediate and thus the two reactions occur sequentially on the surface of the enzyme (Siebert *et al.*, 1957b). This paper constitutes part of a systematic investigation of those regions of the enzyme involved in the two reactions and the relationships between them. It has previously been shown (Colman, 1967, 1968) that alkylation of a single methionyl residue of isocitrate dehydrogenase with iodoacetate results in a greater disruption of the ability of the enzyme to catalyze dehydrogenation than decarboxylation, suggesting that the active sites for these reactions are not identical. A further requirement for enzymatic activity is the dissociated form of an enzyme carboxyl group, as indicated by analyses of pH–rate profiles.

The early work of Lotspeich and Peters (1951) demonstrated a sensitivity of isocitrate dehydrogenase to inhibitors usually assumed to react with sulfhydryl groups, including mercurials and some arsenicals. Siebert *et al.* (1957b) and Plaut (1963) pursued the inhibition of the enzyme by *p*-mercuribenzoate, although these studies were not quantitative. Recently, it has been demonstrated that dinitrophenylation of 1.6–1.8 cysteine residues inactivates gluconate 6-phosphate dehydrogenase, which catalyzes a reaction similar to that of isocitrate dehydrogenase (Rippa *et al.*, 1966). It thus seemed pertinent to explore in detail the role of SH residues in the latter enzyme.

In the present investigation, it will be demonstrated that loss of both the dehydrogenase and decarboxylase functions accompanies modification of isocitrate dehydrogenase by 5,5′-dithiobis(2-nitrobenzoic acid). Modification of one to two reactive SH groups is responsible for changes in the catalytic activity of the enzyme. The rate of inactivation is markedly decreased in the presence of isocitrate or TPNH and manganous sulfate, indicating a specific modification at the active site.

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Experimental Procedure

Materials. Pig heart TPN-dependent isocitrate dehydrogenase was supplied as a solution in 50% glycerol by Boehringer Mannheim Corp. and was further purified tenfold by column chromatography on carboxymethylcellulose, followed by gel filtration on Sephadex G-150, as previously described (Colman, 1968). The resulting protein is homogeneous on electrophoresis and ultracentrifugation, and exhibits a molecular weight of 58,000.

The protein concentration of purified enzyme was determined from its absorbance at 280 mμ, based on a value of 9.10 for $E^{1\%}_{280}$ (Colman, 1968). Enzyme modified by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) displays an altered ultraviolet absorption spectrum. Thus, protein concentration in that case was measured by the biuret reaction with bovine serum albumin as the standard.

Alkylated enzyme was prepared by incubation of 2.3 mg/ml of isocitrate dehydrogenase with 2.8×10^{-2} M iodoacetate at pH 5.8 and 30°, as previously described (Colman, 1968).

All coenzymes and substrates were purchased from Sigma Chemical Co.; Aldrich Chemical Co. supplied 5,5′-dithiobis(2-nitrobenzoic acid); and dithiothreitol (A grade) was obtained from Calbiochem.

Barium oxalosuccinate was synthesized by acid hydrolysis of triethyl oxalosuccinate (K & K Laboratories) as described by Ochoa (1948). The barium salt was stored over calcium sulfate at 5°. Solutions of oxalosuccinate were prepared immediately prior to use.

Kinetic Studies. Unless otherwise indicated, isocitrate dehydrogenase activity was measured at 23° in 0.03 M triethanolamine chloride buffer (pH 7.4) with 1×10^{-4}, 4×10^{-3}, and 2×10^{-3} M TPN, DL-isocitrate, and manganese sulfate, respectively, in a total volume of 1.0 ml (1-cm path-length cuvets). Initial velocities were determined spectrophotometrically at 340 mμ with an expanded-scale recorder (0.1-absorbance full scale) using either a Gilford Model 242 or Cary Model 15 spectrophotometer. In determining Michaelis constants, each substrate was varied in turn, with the remaining substrates being maintained at the concentrations given in this section. In the case of the K_m for isocitrate and TPN, 10-cm path-length cells were employed, and the total volume was 25 ml.

Reductive carboxylase activity was measured spectrophotometrically at 340 mμ and 23° in 0.04 M triethanolamine chloride buffer (pH 7.4) with 2×10^{-4}, 2×10^{-2}, 4×10^{-2}, and 2×10^{-3} M TPNH, α-ketoglutarate, potassium bicarbonate, and manganese sulfate, respectively, in a total volume of 1.0 ml. The potassium bicarbonate stock solution was saturated with CO_2 prior to use.

Oxalosuccinate decarboxylase activity was measured spectrophotometrically at 240 mμ and 23°, in accordance with Grafflin and Ochoa (1950). A total volume of 1.0 ml contained 1.34×10^{-1}, 2.3×10^{-4}, and 2.3×10^{-4} M of KCl, oxalosuccinate, and manganese sulfate, respectively, in 0.2 M sodium acetate buffer (pH 5.5). Oxalosuccinate decarboxylase activity was also assessed by measuring the rate at which protons are taken up following loss of CO_2. A Radiometer automatic titrator equipped with syringe buret (0.5-ml capacity) and a titrigraph were used. A total volume of 2.0 ml contained 1.34×10^{-1} M KCl, 1.2×10^{-3} M $MnSO_4$, and 2.3×10^{-3} M oxalosuccinate in an unbuffered solution adjusted to pH 5.45. The volume uptake of 0.01 M HCl was recorded as a function of time and corrections were made for the rate of nonenzymatic decarboxylation.

Reaction with 5,5′-Dithiobis(2-nitrobenzoic acid). Isocitrate dehydrogenase was incubated with 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1 M triethanolamine chloride buffer (pH 7.7) containing 0.3 M Na_2SO_4, 1 mM EDTA, and 10% glycerol. The number of enzymic SH groups involved was determined from the concentration of 2-nitro-5-mercaptobenzoate as indicated by the increase in absorbance at 412 mμ (Ellman, 1959). Protein and reagent concentrations as well as the temperature are specified for each experiment.

Determination of Extinction Coefficient of Reaction Product of Mercaptoethanol and 5,5′-Dithiobis(2-nitrobenzoic acid). Isocitrate dehydrogenase, after reaction with 5,5′-dithiobis(2-nitrobenzoic acid), exhibits an additional ultraviolet absorption maximum at 323 mμ, as will be described in Results. As a model compound with which to compare the spectrum of the mixed disulfide of the enzyme and 5,5′-dithiobis(2-nitrobenzoic acid), the disulfide product resulting from reaction between mercaptoethanol and 5,5′-dithiobis(2-nitrobenzoic acid) was examined. For this purpose, the absorbances at 412 mμ (peak absorbance for 2-nitro-5-mercaptobenzoate) and 323 mμ (peak absorbance for 5,5′-dithiobis(2-nitrobenzoic acid) and mixed disulfide) were measured for solutions containing a constant concentration of mercaptoethanol and concentrations of 5,5′-dithiobis(2-nitrobenzoic acid) varying from levels less than to greater than the concentration of mercaptoethanol. The absorbance at 323 mμ is the sum of contributions from unreacted 5,5′-dithiobis(2-nitrobenzoic acid), the product, 2-nitro-5-mercaptobenzoate, and the mixed disulfide, the extinction coefficient of which is to be measured. An absorption peak at 323 mμ with a molar extinction coefficient of 1.66×10^4 is characteristic of 5,5′-dithiobis(2-nitrobenzoic acid) itself. The contribution of the residual reagent to the absorbance at 323 mμ can thus be calculated by subtracting from the original 5,5′-dithiobis(2-nitrobenzoic acid) concentration the concentration of 2-nitro-5-mercaptobenzoate produced as measured by the maximum absorbance at 412 mμ (ϵ 1.36×10^4). The concentration of the mixed disulfide is the same as that of the 2-nitro-5-mercaptobenzoate generated.

To determine the extinction coefficient at 323 mμ of 2-nitro-5-mercaptobenzoate in the absence of the mixed disulfide, excess isocitrate dehydrogenase was added to a solution of known 5,5′-dithiobis(2-nitrobenzoic acid) concentration to convert the reagent quantitatively into its products. The incubation mixture was passed through a 1-ml syringe ultrafilter (purchased from Amicon Corp.) equipped with a Diaflo UM-1 membrane which retains solutes of molecular weight greater

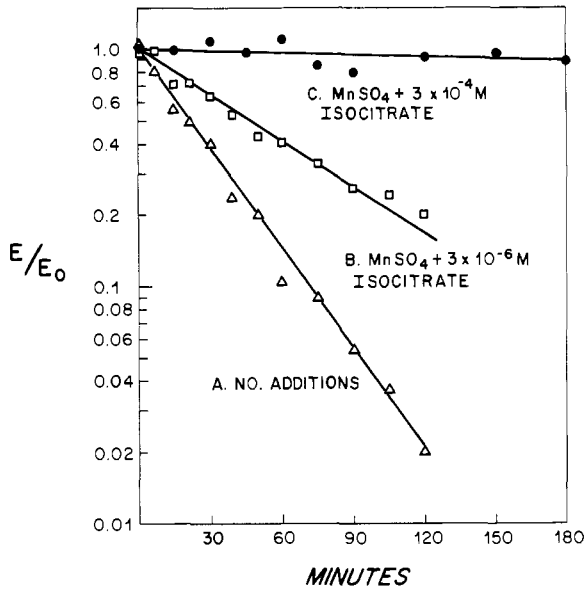

FIGURE 1: Kinetics of inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence and absence of substrate. In these experiments 0.49 mg/ml of isocitrate dehydrogenase was incubated with 2.03×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid) at 25°. Manganese sulfate (2×10^{-3} M) and *threo*-Ds-isocitrate were added as indicated. Aliquots were withdrawn, diluted 20-fold with the same buffer at 0°, and assayed for isocitrate dehydrogenase activity as described in Experimental Procedure. The pseudo-first-order rate constants were calculated from the relationship $\ln E/E_0 = -k_{app}t$, where E is the residual active and E_0 is the total enzyme concentration. The rate constants are 32.0×10^{-3} min^{-1}, 14.8×10^{-3} min^{-1}, and 0.531×10^{-3} min^{-1} for lines A, B, and C, respectively.

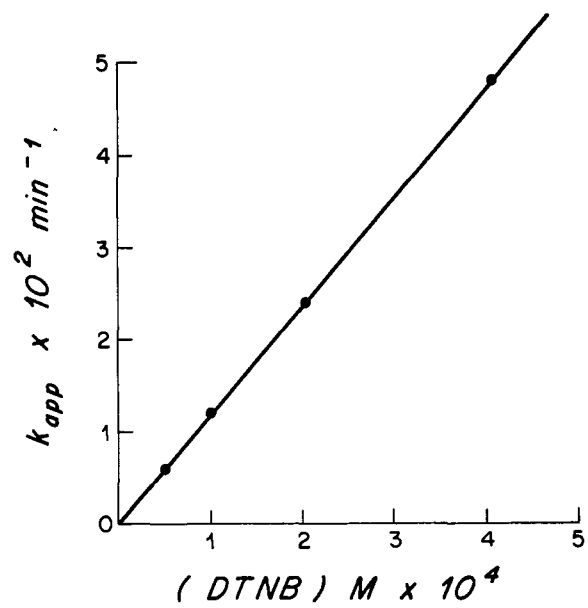

FIGURE 2: Effect of 5,5′-dithiobis(2-nitrobenzoic acid) concentration on the pseudo-first-order rate constant for inactivation. Isocitrate dehydrogenase (0.38 mg/ml) was incubated with 5,5′-dithiobis(2-nitrobenzoic acid), as indicated, at 25°. The pseudo-first-order rate constants for inactivation were obtained as described in Figure 1. The rate constants given in this figure have been corrected for the rate of inactivation of the enzyme extrapolated to zero reagent concentration.

than 10,000, thereby removing the protein-bound mixed-disulfide product from 2-nitro-5-mercaptobenzoate in the filtrate. The spectrum of the filtrate evidenced the 412-mμ peak characteristic of 2-nitro-5-mercaptobenzoate and an extinction coefficient of 4.29×10^3 at 323 mμ. Having determined the concentrations of 5,5′-dithiobis(2-nitrobenzoic acid), 2-nitro-5-mercaptobenzoate, and mixed disulfide, and the extinction coefficients at 323 mμ of the first two, the molar extinction coefficient at 323 mμ for the mixed disulfide formed from mercaptoethanol and 5,5′-dithiobis(2-nitrobenzoic acid) was calculated to be 2.45×10^3.

Results

Inactivation by 5,5′-Dithiobis(2-nitrobenzoic acid)

Protection by Substrates. The TPN-dependent pig heart isocitrate dehydrogenase can be entirely inactivated by incubation with 5,5′-dithiobis(2-nitrobenzoic acid). The concentration of 5,5′-dithiobis(2-nitrobenzoic acid) is in considerable excess over that of the enzyme and therefore pseudo-first-order kinetics are observed, as shown in Figure 1. A 60-fold decrease in the first-order rate constant is produced by the addition to the incubation mixture of 3×10^{-4} M *threo*-Ds-isocitrate and 2×10^{-3} M manganous sulfate. The apparent first-order rate constant for inactivation is directly proportional to the concentration of 5,5′-dithiobis(2-nitrobenzoic acid), over a concentration range from 5×10^{-5} to 4×10^{-4} M, as demonstrated by the linear plot of Figure 2.

The influence of substrates and coenzymes on the apparent first-order rate constant for inactivation at a constant concentration of 5,5′-dithiobis(2-nitrobenzoic acid) is exhibited in Table I. The concentrations of substrates were, in general, chosen to be approximately 20 times their respective Michaelis constants. The exceptions are manganous sulfate, in which case a higher concentration (2 mM) was added in order to exceed the concentration of EDTA (1 mM) in the buffer, and bicarbonate, for which an accurate Michaelis constant has not been determined. Striking protection is afforded by isocitrate or TPNH when added in the presence of manganous sulfate; although isocitrate and reduced pyridine nucleotide are ineffective when added separately. The metal ion produces only a slight decrease in the rate constant when added alone. The other substrates, α-ketoglutarate and bicarbonate, do not effect a significant decrease in the rate of inactivation either in the presence or absence of manganous sulfate. Similarly, the coenzyme TPN does not appear to influence the modification reaction.

Kinetic Analysis of Substrate Protection. Inactivation of isocitrate dehydrogenase by 5,5′-dithiobis(2-nitrobenzoic acid) can be considered as a special case of a mechanism in which an inhibitor competes with substrate for the same site or for different but mutually exclusive sites on an enzyme, but where it is not possible to demonstrate competitive inhibition by the conventional methods because both the association and dis-

sociation reactions of the enzyme and inhibitor are slow. The kinetic scheme is formulated as follows

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \longrightarrow E + P$$

$$E + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI$$

where $K_m = (k_{-1} + k_2)/k_1$ and $K_I = k_{-3}/k_3$. The steady-state assumption is made with respect to the enzyme–substrate complex, but not the enzyme–inhibitor complex; rather, the differential equation involving EI is solved. From this kinetic scheme, the following equation[1] has been derived for the initial rate of reaction of substrate in the presence of inhibitor (Colman, 1962).

$$v = \frac{V_m}{1 + \frac{K_m}{(S)}}\left[1 - \left(\frac{1}{1 + \frac{K_I}{(I)}\left(1 + \frac{(S)}{K_m}\right)}\right) \times (1 - e^{-k_3 R t})\right] \quad (1)$$

where $R = \{K_I + [(I)/(1 + (S)/K_m)]\}$. At $t = 0$, the exponential term is 1 and the velocity equation reduces to the standard Michaelis–Menten expression for initial velocity in the absence of any inhibitor. Subsequently, this velocity is decreased to an extent dependent upon time and upon the concentration of substrate and inhibitor.

This equation should be applicable to a variety of enzymatic reactions. Examples of its usefulness are found in the inhibition of the clostridial enzyme acetoacetate decarboxylase by acetopyruvate (Colman, 1962; Tagaki *et al.*, 1968) and in the inhibition of chymotrypsin by phenyl arsonates (Glazer, 1968).

In examining the effect of substrate on the rate of inactivation of an enzyme by a reagent, as in the case of the modification of isocitrate dehydrogenase by 5,5′-dithiobis(2-nitrobenzoic acid), it is necessary to consider only the exponential term of equation 1, *i.e.*

$$k_{app} = k_3\left[K_I + \frac{(I)}{1 + \frac{(S)}{K_m}}\right] \quad (2)$$

where k_{app} is the apparent rate constant for inactivation in the presence of given concentrations of substrate and inhibitor. Under the conditions of these kinetic experiments, the disulfide-exchange reaction is effectively irreversible and K_I is zero. The inactivation rate is thus expressed as

$$k_{app} = k_3\left[\frac{(I)}{1 + \frac{(S)}{K_m}}\right] \quad (3)$$

Since k_3 is readily measured in the absence of substrate, it is apparent that determination of k_{app} at different concentrations of isocitrate gives an estimate of K_m for the substrate site relevant to protection against 5,5′-dithiobis(2-nitrobenzoic acid). Table II records these constants and demonstrates close agreement between the mean K_m for *threo*-Ds-isocitrate calculated from the rates of inactivation (3.9×10^{-6} M) and the K_m determined directly by the effect of *threo*-Ds-isocitrate on the initial enzymatic rate (4.1×10^{-6} M). Similar results are obtained from the measurement of k_{app} at different concentrations of TPNH, as reported in Table III. The mean K_m for reduced pyridine nucleotide calculated from the inactivation (1.15×10^{-5} M) agrees well with that determined directly (1.2×10^{-5} M). Correspondence between the isocitrate and TPNH binding sites operative in catalysis and protection against 5,5′-dithiobis(2-nitrobenzoic acid) is strongly implied.

Effect on Dehydrogenase and Decarboxylase Activities. Isocitrate dehydrogenase catalyzes the decarboxylation of oxalosuccinate as well as the TPN-dependent oxidation of isocitrate. The data of Siebert *et al.* (1957b) suggest and more recent results (Colman, 1969) support the notion that the rate-determining step in the forward reaction is the dehydrogenation of isocitrate, whereas the slow step in the reverse reaction is the carboxylation

TABLE I: Effect of Substrates on Inactivation by 5,5′-Dithiobis(2-nitrobenzoic acid).[a]

Addition to Reaction Mixture	Inactivation Rate Constant, k_{app} ($\times 10^3$ min^{-1})
None	38.0
2×10^{-3} M $MnSO_4$	24.0
Substrates or coenzymes + 2×10^{-3} M $MnSO_4$	
8×10^{-5} M *threo*-Ds-isocitrate	1.77
2×10^{-4} M TPNH	2.43
1×10^{-2} M α-ketoglutarate	19.7
3×10^{-2} M $KHCO_3$	24.2
3×10^{-2} M $KHCO_3$ + 1×10^{-2} M α-ketoglutarate	24.0
1×10^{-4} M TPN	26.0
Substrates or coenzymes alone	
8×10^{-5} M *threo*-Ds-isocitrate	8.33
2×10^{-4} M TPNH	33.3
1×10^{-2} M α-ketoglutarate	39.3
3×10^{-2} M $KHCO_3$	33.1
1×10^{-4} M TPN	34.0

[a] In these experiments, isocitrate dehydrogenase (0.38 mg/ml) was incubated at 25° with 2.03×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid). Substrates were added as indicated. Aliquots were taken, immediately diluted 25-fold with the same buffer at 0°, and assayed for isocitrate dehydrogenase activity as described in Experimental Procedure. The apparent rate constants were calculated as described in Figure 1.

[1] The derivation of this equation is available upon request.

TABLE II: Michaelis Constant for Isocitrate Calculated from Inactivation Rate by 5,5′-Dithiobis(2-nitrobenzoic acid).[a]

Addition to Reaction Mixture	Inactivation Rate Constant, k_{app} ($\times 10^3$ min^{-1})	Calcd K_m of Isocitrate (μM)[b]
None	32.0	
2 mM $MnSO_4$		
$+3 \times 10^{-6}$ M *threo*-Ds-isocitrate	14.8	2.6
$+1 \times 10^{-5}$ M *threo*-Ds-isocitrate	9.55	4.2
$+3 \times 10^{-5}$ M *threo*-Ds-isocitrate	3.53	3.7
$+3 \times 10^{-4}$ M *threo*-Ds-isocitrate	0.531	5.0
		Mean 3.9

[a] These experiments were conducted as described in Figure 1. The Michaelis constants for *threo*-Ds-isocitrate were calculated from the rates of inactivation of enzyme by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence and absence of *threo*-Ds-isocitrate and $MnSO_4$ in accordance with eq 3. [b] The experimental Michaelis constant for *threo*-Ds-isocitrate, measured as described in Experimental Procedure, was 4.1 μM.

TABLE III: Michaelis Constant for TPNH Calculated from Inactivation Rate by 5,5′-Dithiobis(2-nitrobenzoic acid).[a]

Addition to Reaction Mixture	Inactivation Rate Constant, k_{app} ($\times 10^3$ min^{-1})	Calcd K_m of TPNH[b] ($\times 10^5$ M)
None	38.0	
2 mM $MnSO_4$		
$+2.2 \times 10^{-5}$ M TPNH	11.1	0.92
$+6.2 \times 10^{-5}$ M TPNH	5.05	0.95
$+2.3 \times 10^{-4}$ M TPNH	2.43	1.58
		Mean 1.15

[a] These experiments were conducted as described in Table I. The Michaelis constants for TPNH were calculated from the rates of inactivation of enzyme by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence and absence of TPNH and $MnSO_4$, in accordance with eq 3. [b] The experimental Michaelis constant for TPNH, measured as described in Experimental Procedure, was 1.2×10^{-5} M.

of α-ketoglutarate. A distinct role for a sensitive amino acid in dehydrogenation as compared with decarboxylation would therefore be reflected in different rates of inactivation as measured by the over-all forward and reverse reactions. The residual isocitrate dehydrogenase and reductive carboxylase activities were thus measured as a function of time of incubation with 1.06×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid) at pH 7.7 and 25°. The pseudo-first-order rate constants for inactivation were almost the same: 0.021 min^{-1} when assessed by the change in dehydrogenase and 0.018 min^{-1}, by the change in reductive carboxylase activity.

Table IV shows the results of a direct comparison of isocitrate dehydrogenase activity with the ability to catalyze the decarboxylation of oxalosuccinate as measured spectrophotometrically or by the uptake of protons at pH 5.5. It is apparent that the two functions of the enzyme are equally affected by the disulfide-exchange reaction.

Kinetic Properties of Partially Inactivated Enzymes. An altered affinity of an intrinsically active enzyme for its substrates is not the cause of the observed loss of activity upon modification by 5,5′-dithiobis(2-nitrobenzoic acid). Enzymes of different residual activities were prepared by incubation with 2.54×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid) for varying time intervals, followed by removal of excess reagent by gel filtration on Sephadex G-25. The Michaelis constants of the preparations for isocitrate, α-ketoglutarate, TPN, TPNH, and $MnSO_4$ were measured. As shown in Table V, no significant differences were noted between these kinetic parameters for the native and modified enzymes, except for an increase in the Michaelis constant for TPNH which is of insufficient magnitude to account for the observed decline in catalytic activity. The measured kinetic parameters for the active enzyme produced by modification in the presence of isocitrate and manganese sulfate are also unaltered, although three SH groups have reacted in this enzyme (Table VI).

Similarly, loss of measured activity under the usual assay conditions cannot be attributed to a shift in the pH–rate profile for the modified enzyme. The pH dependence of the isocitrate dehydrogenase reaction was unchanged in these preparations as compared with that of native enzyme (Colman, 1968).

Number of Sulfhydryl Groups Modified

Titration of Reactive SH Groups. METHOD I. The number of reactive SH groups in the native enzyme in the presence and absence of substrates was determined by the method of Ellman (1959) involving direct spectrophotometric measurement of the 2-nitro-5-mercaptobenzoate released upon incubation with 5,5′-dithiobis(2-nitrobenzoic acid), as shown in Table VI (Method I). Approximately five enzyme SH groups are available for reaction with 5,5′-dithiobis(2-nitrobenzoic acid) under these conditions, as a result of which activity is lost. The absorbance at 412 mμ becomes constant within 120 min, indicating that the reaction has reached completion, and no change is noted upon prolonging the incubation to 18 hr. When the enzyme is first denatured in 0.2% sodium dodecyl sulfate, however, 13 SH groups participate in the disulfide-exchange reaction, suggesting that the native structure determines the lack of reactivity of 8 SH groups.

TABLE IV: Effect of 5,5′-Dithiobis(2-nitrobenzoic acid) on Dehydrogenase and Decarboxylase Activities.[a]

5,5′-Dithiobis(2-nitrobenzoic acid)(M × 10^4)	Dehydrogenase Activity	Decarboxylase Activity	
	Spectrophotometric (%)	Spectrophotometric (%)	Titrimetric (%)
0	100	100	100
1.02	66	67	72
2.54	26	25	27

[a] In these experiments isocitrate dehydrogenase (2 mg/ml) was incubated with 5,5′-dithiobis(2-nitrobenzoic acid) as indicated for 1 hr, at 25°. Assays were conducted as described in Experimental Procedure.

TABLE V: Michaelis Constants for Substrates for Native and Partially Active Enzymes.[a]

Residual Act. (%)	$K_{DL\text{-isocitrate}}$ (μM)	$K_{\alpha\text{-ketoglutarate}}$ (mM)	K_{TPN} (μM)	K_{TPNH} (μM)	K_{Mn} (μM)
100 (native)	5.7	0.51	4.6	12.0	4.3
100 (protected with isocitrate + $MnSO_4$)	5.6		6.1		
50	5.9	0.59	4.0		
36				27.0	4.0
15	6.4		5.7		

[a] Isocitrate dehydrogenase was incubated for varying time intervals with 2.54 × 10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid) at room temperature. The protected enzyme was incubated for a time sufficient to reduce activity to 5% in the absence of substrates. At the end of the selected interval, the reaction was terminated by gel filtration on Sephadex G-25 equilibrated at 0° with 0.1 M triethanolamine chloride buffer (pH 7.7) containing 0.3 M Na_2SO_4, 1 mM EDTA, and 10% glycerol. The Michaelis constants were determined at pH 7.4, as described in Experimental Procedure, on the same day as the partially active enzyme was prepared. Only the Michaelis constant for $MnSO_4$ was measured at pH 5.5 and in this case EDTA was deleted from the buffer.

In attempting to relate the number of modified SH groups with the effect of the reagent on activity in the presence and absence of substrates the assumption is made that the substrates will exert an influence on the reactivity of particular SH groups rather than on all of them. Those SH groups whose reactivity is unaffected by the substrates added would be expected to react completely with 5,5′-dithiobis(2-nitrobenzoic acid) within 120 min, as is the case for the enzyme in the absence of substrates. In support of this assumption, the rate constant for reaction with the SH groups listed in Table VI is essentially the same, whether or not substrates are present; only the number of measurable groups changes when substrates are included. Those groups whose reactivity is decreased by substrates would be expected to react at a rate that is no greater than 5% of that observed in the control, in accordance with the presence of substrate concentrations at least 20 times the value of K_m. Any effect that substrates exert on the rate constant for reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with these particular SH groups will thus be reflected in an altered number of groups modified at 120 min.

On this basis, the number of reactive SH groups is unchanged in the presence of TPN or $MnSO_4$, corresponding to the lack of effectiveness of these compounds in preventing inactivation by 5,5′-dithiobis(2-nitrobenzoic acid). When isocitrate or α-ketoglutarate are added to the reaction mixture alone, there is a slight increase in the number of SH groups available, perhaps reflecting a small conformational change induced or stabilized by these substrates. The resultant enzymes are inactive. The inclusion of TPNH alone in the incubation mixture yields an inactive enzyme which exhibits a decrease of approximately one reactive SH group. This decrease may also reflect a conformational alteration of the enzyme caused by the coenzyme, but is not directly related to the activity change.

In contrast, in the presence of both isocitrate and $MnSO_4$, or TPNH and $MnSO_4$, conditions under which activity is substantially retained, there is a decrease of approximately two reactive SH residues. A maximum of two SH groups is thus implicated in the loss of activity upon modification.

It is not possible to measure directly the number of reactive SH groups in the presence of high concentrations of metal ion, since 2 × 10^{-3} M manganous sulfate interferes with the spectrophotometric measurement (although 2 × 10^{-4} M does not), presumably by complexing with the 2-nitro-5-mercaptobenzoate generated. Thus, another method was sought in which the number of altered SH groups could be determined after removal of excess substrate and reagent.

Determination of Disulfide Produced. METHOD II.

TABLE VI: Determination of Reactive SH Groups in the Presence of Substrate.[a]

Additions to Reaction Mixture	Method I Direct	Method II After Dialysis
I. Native enzyme		
None	4.97	4.91
2×10^{-3} M $MnSO_4$ + 4×10^{-3} M isocitrate		2.89
2×10^{-4} M $MnSO_4$ + 4×10^{-3} M isocitrate	2.64	
2×10^{-3} M $MnSO_4$		4.94
2×10^{-4} M $MnSO_4$	4.77	
2×10^{-4} M TPNH	4.02	
2×10^{-4} M TPNH + 2×10^{-4} M $MnSO_4$	3.28	
1×10^{-4} M TPN	4.99	
4×10^{-3} M isocitrate	5.42	
1×10^{-2} M α-ketoglutarate	5.49	
II. Alkylated enzyme		
None	4.21	
2×10^{-4} M $MnSO_4$ + 4×10^{-3} M isocitrate	3.19	

[a] In method I, isocitrate dehydrogenase was allowed to react at 25° with 2.54×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid) for 120 min, at which time the absorbance at 412 mμ was used to calculate the reactive SH content. Substrates were added as indicated. In experiments involving $MnSO_4$, EDTA was deleted from the buffer. In method II, enzyme, reacted with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence or absence of substrates, was first dialyzed at 4° against 0.1 M triethanolamine chloride buffer (pH 7.7) containing the substrate used in the experiment and 1 mM EDTA, 0.3 M Na_2SO_4, and 10% glycerol, followed by dialysis against the buffer without substrate. Protein concentration was measured by the biuret method. The mercaptide content was determined from the absorbance at 323 mμ as described in the text.

Isocitrate dehydrogenase was incubated with 2.54×10^{-3} M 5,5′-dithiobis(2-nitrobenzoic acid) for 2 hr at 25°, following which it was dialyzed exhaustively against triethanolamine chloride buffer (pH 7.7) as described in Table VI to remove excess reagent and 2-nitro-5-mercaptobenzoate. The total number of unreacted SH groups remaining in this inactive dialyzed enzyme was measured by conventional spectrophotometric titration at 412 mμ with 5,5′-dithiobis(2-nitrobenzoic acid) at pH 7.7 in the presence of the denaturant, 0.2% sodium dodecyl sulfate. Under these conditions, the modified enzyme exhibited 8.29 SH groups, whereas untreated enzyme contained 13.20 residues. The difference of 4.91 must represent those SH groups which had reacted with 5,5′-dithiobis(2-nitrobenzoic acid) prior to dialysis. This value corresponds closely with that of 4.97 obtained by direct spectrophotometric titration of the enzyme (Table VI, method I).

The ultraviolet absorption spectrum, after dialysis, of this enzyme containing 4.91 modified SH groups is shown in Figure 3 together with that of the same concentration of native enzyme. A decrease in the absorbance of the modified enzyme in the 280-mμ region and an increase below 260 mμ is noted; but more important, for these purposes, is the marked increase in absorbance at about 310 mμ, where the native enzyme is transparent. Using the value of 4.91 altered SH groups, an average extinction coefficient at 323 mμ of 2.62×10^3/modified SH group was calculated. Implicit in this calculation is the assumption that the product of each protein SH group exhibits the same extinction coefficient, regardless of its particular environment. The validity of this assumption is not known; however, this extinction coefficient compares favorably with that determined for the mixed disulfide formed from mercaptoethanol and 5,5′-dithiobis(2-nitrobenzoic acid) (2.45×10^3) as described in Experimental Procedure. The close correspondence between these two extinction coefficients suggests that the enzymic derivatives behave as normal aliphatic disulfides.

Reactive SH of Alkylated Enzyme. It has previously been shown that treatment of isocitrate dehydrogenase with iodoacetate at pH 5.5 leads to the specific alkylation of a single methionyl residue in the active site (Colman, 1967, 1968). No difference had been observed between the total SH content of the native and alkylated enzyme as measured by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) after denaturation in 0.2% sodium dodecyl sulfate. In order to determine whether the presence of the carboxymethyl group on an essential methionyl residue influences the reactivity of SH groups at the active site, enzyme which had been totally inactivated with iodoacetate was examined under the nondenaturing conditions of method I (Table VI). In the absence of substrates, the alkylated enzyme exhibited approximately one less reactive SH group than observed for the native enzyme. In the presence of isocitrate and manganese sulfate, the alkylated enzyme, which has been shown to bind these substrates (R. F. Colman, manuscript in preparation), contained three reactive SH groups, as does the native enzyme. The effect of substrates on the alkylated enzyme is to decrease the number of reactive SH groups by one, rather than by two, as in the case of native enzyme. Carboxymethylation of methionine thus blocks one of the SH groups normally protected by isocitrate and manganous ion. Close proximity between an active site SH and methionine is thereby implied.

It is notable that in these experiments the rate constant for reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with the four or three SH groups of the alkylated enzyme was the same as that for reaction with five SH groups of native enzyme. Hence, these results cannot be interpreted as resulting from a decreased reaction rate at all five SH groups.

Temperature Dependence of Inactivation and SH Reaction. The relationship between the rate of reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with enzyme SH

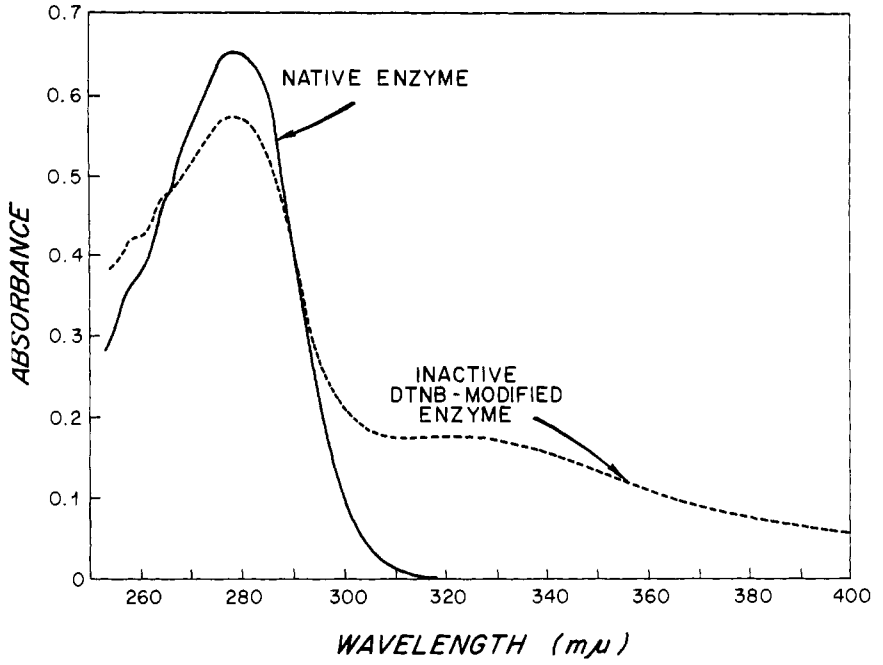

FIGURE 3: Spectrum of native isocitrate dehydrogenase and enzyme modified with 5,5′-dithiobis(2-nitrobenzoic acid). Native enzyme, 0.77 mg/ml. Modified enzyme, 0.77 mg/ml. Enzyme was incubated with 2.54×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid) at 25° until the absorbance at 412 mμ was constant and then dialyzed against 0.1 M triethanolamine chloride buffer (pH 7.7) containing 1 mM EDTA, 0.3 M Na_2SO_4, and 10% glycerol. Protein concentrations were measured by the biuret reaction.

groups and the rate of inactivation was examined over the temperature range from 15 to 30° in order to determine whether the one to two residues involved in catalytic activity differed in reactivity from the other SH groups. The pseudo-first-order rate constants for reaction with SH groups were measured both by the increase in absorbance at 412 mμ, and by the decrease in isocitrate dehydrogenase activity, and are recorded in Table VII. It is apparent that the rate of inactivation is always slightly higher than the average rate of reaction with five SH groups in the enzyme. However, the ratio of the rate constants is never greater than 1.29, and, furthermore, it does not vary with temperature. It thus appears that although five SH groups of isocitrate dehydrogenase are more "available" than the other eight, there is little distinction in reactivity among these five residues.

Secondary Inactivation and Reactivation. Isocitrate dehydrogenase which has been partially inactivated with 5,5′-dithiobis(2-nitrobenzoic acid) and then separated from excess reagent by gel filtration on Sephadex G-25 is subject to a secondary inactivation process occurring over a period of days at 4°. For example, an isolated 49% active 5,5′-dithiobis(2-nitrobenzoic acid)–enzyme was reduced to 11% of its residual activity after 4 days. The loss of activity is accompanied by an increase in absorbance at 412 mμ, signified by a yellow appearance of the solution. These observations suggest that a disulfide-exchange reaction between the active site mixed disulfide and other enzyme SH groups results in the secondary loss of activity and release of free 2-nitro-5-mercaptobenzoate.

Instead of further inactivation, a slow increase in enzymatic activity is noted when 2.8 mM mercaptoethanol is added to solutions of partially active enzymes from which excess reagent has been removed. For example, an enzyme of 17% residual activity was restored to 65% of the original activity after incubation with mercaptoethanol for 3 days. The addition of mercaptoethanol is accompanied by a rapid release of 2-nitro-5-mercaptobenzoate, as observed by the increase in absorbance at 412 mμ to a constant value in less than 5 min, followed by the slower restoration of enzymatic

TABLE VII: Temperature Dependence of Reaction Rate of 5,5′-Dithiobis(2-nitrobenzoic acid) with Isocitrate Dehydrogenase as Measured by Inactivation and Reaction with SH Groups.[a]

Temp (°C)	Inactivation Rate Constant, k_{in} ($\times 10^2$ min^{-1})	SH Rate Constant, k_{SH} ($\times 10^2$ min^{-1})	k_{in}/k_{SH}
15	1.16	0.909	1.28
20	2.18	1.69	1.29
25	4.83	4.12	1.17
30	7.96	6.48	1.23

[a] Enzyme (0.23 mg/ml) was incubated with 2.54×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid) at the temperature indicated. The pseudo-first-order rate constants for reaction with SH groups were derived from a continuous record of the absorbance at 412 mμ as a function of time using a Cary Model 15 equipped with a thermostatable cell adaptor. Aliquots of the reaction mixture were withdrawn, diluted 20-fold with the same buffer at 0°, and assayed for isocitrate dehydrogenase activity as described in Experimental Procedure. The rate constants for inactivation were calculated from pseudo-first-order plots of the residual enzymatic activity as a function of time, as described in Figure 1.

activity. An inactive unsymmetrical disulfide of the enzyme SH groups and mercaptoethanol may initially replace the inactive mixed disulfide of 2-nitro-5-mercaptobenzoate and the enzyme SH group, with subsequent reduction by excess mercaptoethanol to regenerate the essential SH group. Alternatively, the rapid release of 2-nitro-5-mercaptobenzoate may precede a slow conformational change essential for reactivation.

The effect of mercaptoethanol affords another method for quantification of the SH groups which have been modified by 5,5′-dithiobis(2-nitrobenzoic acid) since the concentration of 2-nitro-5-mercaptobenzoate released can be measured. This approach is similar in principle to the determination of protein SH groups by reaction under acidic conditions with nitrophenylsulfenyl chloride, followed by release of the nitrothiophenolate moiety in alkaline solution (Fontana *et al.*, 1968).

In contrast to mercaptoethanol, 2 mM dithiothreitol does not markedly promote reactivation, although it releases free thiophenolate ion from the mixed disulfide. Its presence does, however, prevent secondary inactivation of 5,5′-dithiobis(2-nitrobenzoic acid)-modified enzymes, so that partially active preparations to which dithiothreitol has been added are relatively stable in activity.

Effect on Molecular Size. The molecular size of isocitrate dehydrogenase is not significantly altered by treatment with 5,5′-dithiobis(2-nitrobenzoic acid), as determined by gel filtration on Sephadex G-150. In these experiments, native enzyme was applied to a column (2.3 × 26.8 cm) equilibrated with 0.1 M triethanolamine chloride buffer (pH 7.7) containing 0.3 M Na_2SO_4, 1 mM EDTA, and 10% glycerol. The Stoke's radius of native isocitrate dehydrogenase was determined as 3.90 Å, using the procedure described by Ackers (1964), with cytochrome *c*, chymotrypsin, bovine serum albumin, lactate dehydrogenase, and Dextran Blue for column calibration. Isocitrate dehydrogenase, after incubation with 5,5′-dithiobis(2-nitrobenzoic acid) to produce a preparation of either 41 or 9% residual activity, was applied to the same calibrated column. A single protein peak without appreciable broadening was observed at 49.7 ml, as compared with 49.8 ml for the untreated enzyme.

Effect on Electrophoretic Mobility. Inactive modified enzyme, in which five SH groups have reacted with 5,5′-dithiobis(2-nitrobenzoic acid), exhibited a single band on cellulose acetate electrophoresis at 200 V for 2 hr in 0.02 M triethanolamine chloride buffer (pH 7.05). There is a net positive charge on the enzyme at this pH (Colman, 1968), and thus, as expected, the introduction of negatively charged 2-nitro-5-mercaptobenzoyl groups results in a decrease in the mobility of the enzyme.

Discussion

An essential role in catalysis can be attributed to a limited number of SH groups of isocitrate dehydrogenase as a result of the experiments here reported. In the absence of substrates, 5,5′-dithiobis(2-nitrobenzoic acid) reacts with five SH groups of isocitrate dehydrogenase and the resultant enzyme is completely inactive. In the presence of isocitrate or TPNH and manganous sulfate, approximately three SH groups react and activity is retained. A maximum of two SH groups is thus responsible for the loss of activity upon modification. These data do not distinguish between the possibilities, on the one hand, that both residues are intimately involved in the enzymatic reaction or, on the other hand, that only one SH group participates directly in catalysis but that a second is close to the active site and is fortuitously masked by the substrate and coenzyme. It is apparent, however, that in the inactive enzyme produced by alkylation of a single essential methionyl residue with iodoacetate, one of the two SH groups normally protected by substrate is blocked from reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Table VI, section II). A relationship between the active site methionyl residue and one of these sulfhydryl groups is thereby established.

Inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) is the result of a specific reaction rather than generalized denaturation, since enzyme incubated under the same conditions but in the absence of reagent remains fully functional and since substrates protect against loss of activity. No change is noted in the molecular weight of the modified enzyme. Furthermore, reaction with 5,5′-dithiobis(2-nitrobenzoic acid) does not impair the ability of isocitrate dehydrogenase to combine with specific antibody to native enzyme (R. F. Colman, manuscript in preparation).

The requirements for protection against inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) are quite stringent: metal ion must be present together with isocitrate, or with TPNH. The postulate that manganous ions are necessary for binding of the substrate does not entirely account for the results. Isocitrate in the absence of metal ion has been found to protect against inactivation by iodoacetate (Colman, 1968); similarly, TPNH in the absence of metal ion decreases by one the number of SH groups reactive toward 5,5′-dithiobis(2-nitrobenzoic acid) (Table VI). The substrate and coenzyme alone are thus capable of binding to the enzyme, although some influence of manganous sulfate on the strength of binding of isocitrate is not excluded.

The substrate, α-ketoglutarate, which functions kinetically as a competitive inhibitor of isocitrate in the dehydrogenase reaction (R. F. Colman, unpublished observations), exerts no protection against 5,5′-dithiobis(2-nitrobenzoic acid) either in the presence or absence of metal ion. However, α-ketoglutarate does provide partial protection against inactivation by iodoacetate, albeit less complete than that exhibited by isocitrate (Colman, 1968). In order to resolve these apparent discrepancies, overlapping but not idential sites may be postulated in which the site for isocitrate includes certain amino acid residues in common with that for α-ketoglutarate, but other amino acid residues which interact specifically with isocitrate. It is possible that the β-carboxylate group of isocitrate, which is lacking in α-ketoglutarate, interacts with or influences the reactivity of those activity-dependent SH groups which are attacked by 5,5′-dithiobis(2-nitrobenzoic acid), and furthermore, that manganous ions are involved in this

interaction. Since isocitrate and α-ketoglutarate both protect against iodoacetate inactivation in the absence of $MnSO_4$, it has previously been proposed that the two substrates interact with the enzyme in the vicinity of the essential methionyl residue (Colman, 1968). The implication is that neither the β-carboxylate group of isocitrate nor manganous ions are essential for this interaction with methionine.

This concept of overlapping sites has previously been applied to binding of purine nucleotides by glutamate dehydrogenase (Colman and Frieden, 1966). In that case, inhibitory and activating purine nucleotides compete kinetically, but their binding is affected differently by acetylation of the enzyme. A similar situation has also been found to exist in the binding of cupric and zinc ions by myoglobin (Banaszak *et al.*, 1965). These two ions compete kinetically for binding, but it has been shown by X-ray diffraction that the separate sites for Cu^{2+} and Zn^{2+} include two of the same amino acids and one residue distinctive for each. Indeed, the existence of overlapping sites on enzymes may be much more common than has heretofore been appreciated and may in part account for the diverse effects on activity exerted by compounds which apparently compete for the same site.

The observation of a decreased rate of chemical modification in the presence of a substrate does not necessarily imply that the protecting substrate binds directly to the group on the enzyme that is normally susceptible to modification. A plausible alternate interpretation is that binding of the particular substrate initiates a change or stabilizes a conformational state in which the susceptible amino acid residues are less reactive toward the reagent. The model of overlapping binding sites for isocitrate and α-ketoglutarate is consistent with either a direct or an indirect effect of substrates on the reactivity of 5,5′-dithiobis(2-nitrobenzoic acid)-sensitive groups. It is possible, for example, that the association of isocitrate with its specific portion of the substrate binding sites effects a conformational change whereas the binding of α-ketoglutarate does not. Significant protection against inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of metal ion is provided by reduced coenzyme as well as by the structurally unrelated isocitrate; and interpretation of protection resulting *indirectly* from a conformational change stabilized either by TPNH or isocitrate perhaps facilitates an explanation of these observations.

It is notable that the Michaelis constants for *threo*-D_S-isocitrate and TPNH as measured by protection against 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of $MnSO_4$ are essentially identical with the values obtained by the usual kinetic methods which examine the effect of substrate or coenzyme concentration on the rate of the enzymatic reaction (Tables II and III). These results suggest that the Michaelis constant for isocitrate and TPNH are true dissociation constants and that the binding of isocitrate and TPNH are not, respectively, influenced by the coenzymes and substrates. Ramakrishna and Krishnaswamy (1966) have presented evidence which suggest the formation of an isocitrate dehydrogenase–CO_2 complex, and Fisher and Cross (1966) have proposed the existence of enzyme–TPNH complex in the absence of substrates. Taken together, these data point to a mechanism for isocitrate dehydrogenase involving random order of addition of substrates to the enzyme. The kinetics of the TPN-dependent isocitrate dehydrogenase reaction appears to conform to this mechanism (Cleland, 1967).

Also consistent with the random-order mechanism are the changes in the number of SH groups available to 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of the substrates, isocitrate and α-ketoglutarate, and the coenzyme, TPNH (Table VI). The small increase in the number of reactive SH groups in the presence of the substrates may reflect a conformational change in the protein as these compounds are bound. The differences in the available SH groups in the presence of isocitrate or TPNH when metal ion is also added as compared with the case where no $MnSO_4$ is present points up the distinction between the mode of binding under these two conditions. The decrease of approximately one reactive SH group in the presence of TPNH may result from a different conformational change in the enzyme produced by the binding of this coenzyme. Coenzyme-induced conformational changes have previously been proposed by Rose (1960) to account for the requirement of TPNH for the exchange of tritium in the solvent with one of the hydrogens on the β-carbon atom of α-ketoglutarate.

The five enzyme SH groups normally available to 5,5′-dithiobis(2-nitrobenzoic acid) in the absence of substrates do not differ markedly in their reactivity, as has been noted (Table VII), although one to two are actually involved in the catalytic function of the enzyme. This contrasts with the general notion that those functional groups in the active site are normally among the most reactive of their class, as exemplified by the essential SH groups of papain (Smith and Kimmel, 1960) and ficin (Holloway *et al.*, 1964). However, it is known that SH compounds which exhibit unusual reactivity in one type of reaction may be unremarkable in another type of reaction. The SH group of ficin, for example, is twenty times as reactive as most simple thiols in displacement reactions, but adds more slowly to *N*-ethylmaleimide than does cysteine (Holloway *et al.*, 1964). It is possible that the lack of distinction of the activity-dependent SH groups in the disulfide-exchange reaction may be unrepresentative of its reactivity in the isocitrate dehydrogenase reaction.

Inactivation of the enzyme by 5,5′-dithiobis(2-nitrobenzoic acid) cannot be ascribed to an altered pK of an essential dissociable group, since the pH dependence of the isocitrate dehydrogenase reaction was the same in native and partially active preparations. Similarly, loss of activity is not due to the weaker binding of substrates by an intrinsically *active* modified enzyme, since the Michaelis constants of partially active enzymes are essentially the same as those of native enzyme. The measurable activity in these partially active enzyme preparations in fact probably represents that of unmodified enzyme and therefore these experiments do not allow one to distinguish between inactivation caused by inability to bind substrates and inactivation

caused by disruption of a step subsequent to binding. These questions are currently under active investigation.

The ability of the enzyme to catalyze oxidative decarboxylation of isocitrate, reductive carboxylation of α-ketoglutarate, and decarboxylation of oxalosuccinate are all equally affected by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). The SH groups thus appear to be involved in both the dehydrogenase and decarboxylase functions of the enzyme. These results contrast with the observation that alkylation of the methionyl residue disrupts primarily the dehydrogenase reaction (Colman, 1968). The concept of overlapping sites discussed with reference to the α-ketoglutarate and isocitrate binding sites is applicable in this situation as well. The active center of an enzyme, in particular one which catalyzes two or more mechanistically distinct reactions, can be considered to include certain amino acid residues that participate only in one partial reaction, other amino acid residues which are exclusively involved in the second partial reaction, and a third group of residues which bridge the two regions of the active site and are essential for both reactions. This scheme is consistent with the current understanding of isocitrate dehydrogenase and may lead to the formulation of a reasonable mechanism for its catalytic reactions.

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