Functional Groups of Diphosphopyridine Nucleotide-linked
Isocitrate Dehydrogenase from Bovine Heart

STUDIES OF CYSTEINE RESIDUES*

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SUMMARY

DPN-linked isocitrate dehydrogenase from bovine heart contains 6 half-cystine residues per subunit of molecular weight of 42,000. All of these residues are present as cysteine since six sulfhydryl groups per subunit can be modified by 5,5'-dithiobis(2-nitrobenzoate) (DTNB). Spectrophotometric measurements indicate that the modification of three thiol groups which react preferentially with DTNB is directly proportional to loss of activity, up to 70% inactivation. 2-Mercaptoethanol and dithiothreitol completely restored activity of enzyme preparations where less than two sulfhydryl groups per subunit had been modified by DTNB, but less than 10% reversal was obtained when more than four sulfhydryl groups had been modified. No reversal with Na2S04 and only partial reversal with KCN is obtained, even though both reagents release 2-nitro-5-mercaptobenzoate in stoichiometric amounts from the modified enzyme. Manganese isocitrate and, to a lesser extent, DPN+ and DPNH protect the enzyme against inactivation by DTNB. In the presence of 0.8 M (NH4)2S04, one sulfhydryl group per subunit can be modified by DTNB and in the presence of manganese isocitrate, one additional group can be modified with full retention of activity. These results suggest that only one of the three reactive sulfhydryl groups is required for activity. The pH inactivation profile with DTNB shows that the activity-related thiol group has a pK of 7.6. Increasing electrolyte concentration does not affect the rate of enzyme inactivation by DTNB; however, it does increase the reactivity of a sulfhydryl group which is not required for activity and decreases the protection given by manganese isocitrate. These results suggest that high salt concentrations interfere with binding of manganese isocitrate to the enzyme, possibly at an active center amino group (Fan, C. C., and Plaut, G. W. E. (1974) Biochemistry 13, 45-59). Lowering the temperature of the preincubation mixture from 23 to 0°C decreases the rate of inactivation by DTNB by about 50% and almost abolishes the protection given by manganese isocitrate. In addition to DTNB, the enzyme is inactivated by p-chloromercuribenzenesulfonate, N-ethylmaleimide, iodoacetate, and iodoacetamide. Manganese isocitrate partially protects against inactivation by these sulfhydryl group modifying agents.

It was shown previously that DPN-linked isocitrate dehydrogenase (three-d, isocitrate + DPN+ → α-ketoglutarate + CO2 + DPNH + H+; EC 1.1.1.41) from bovine heart and porcine liver was inhibited by low concentrations of p-mercuribenzenesulfonate and Ellman's reagent (1-3). The inactivation by 67 μM p-mercuribenzenesulfonate could be partially prevented by a combination of isocitrate and Mn++. DPN+ or ADP did not afford such protection. In this paper, the reaction of bovine heart enzyme with a number of sulfhydryl group modifying agents, especially 5,5'-dithiobis(2-nitrobenzoate) is reported. The protection by substrates and cofactors against these inactivating agents is also described.

The present investigation demonstrates that 3 of the 6 cysteine residues per subunit of enzyme can be modified without drastic alteration of protein structure and that loss of enzyme activity correlates with modification of one of these three reactive thiol groups. Manganese isocitrate protects this activity-related sulfhydryl group against modification by DTNB.1

EXPERIMENTAL PROCEDURES

Materials

Iodoacetic acid, iodoacetamide, p-chloromercuribenzenesulfonic acid, DL-isocitric lactone, Hepes, and nucleotides were purchased from Sigma. 5,5'-Dithiobis(2-nitrobenzoic acid) and N-ethylmaleimide were from Aldrich, 2-mercaptoethanol and EDTA were from Eastman, dithiothreitol was from Calbiochem, and inorganic salts of analytical reagent grade were from Mallinckrodt Chemical Works.

DPN-linked isocitrate dehydrogenase was purified from bovine heart by the method of Giorgio et al. (4). It showed a single band in polyacrylamide electrophoresis and had a specific activity of 28,000 units of DPN+ reduced per min per mg of protein at 25°C under the conditions of assay reported previously (4). Protein was determined as described previously (5).

1 The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoate); TNB, 2-nitro-5-thiobenzoate; Hepes, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid.
Methods

Modification and Inactivation—Enzyme (approximately 0.01 mM) was incubated with various reagents as specified for each experiment; protection against inhibition was described previously (6). Aliquots (10 μl or less) were removed from the preincubation mixtures at appropriate time intervals and activity was measured in a final volume of 1.0 ml in the standard assay (4). Inactivation of enzyme activity was expressed as apparent pseudo-first order rate constants (kapp) as in previous studies (5, 6). Modification of enzyme sulphydryl groups by DTNB was determined by following the increase of absorbance at 412 nm of 2-nitro-5-mercaptobenzoate liberated (ε412 = 13,600 liters mole⁻¹ cm⁻¹) (7).

Incorporation of radioactivity from N[2,3-14C]methylmaleimide into protein was determined after terminating the reaction with 2-mercaptoethanol followed by extensive dialysis.

Modification of sulphydryl groups is expressed on the basis of an enzyme subunit molecular weight of 42,000 (4).

Reactions of DTNB-treated Enzyme with Nucleophiles—Enzyme was incubated with DTNB under conditions specified. At appropriate time intervals, modification of sulphydryl groups was determined spectrophotometrically at 412 nm and enzyme activity was determined; nucleophile then was added and incubation was continued until enzyme activity became constant. Activities before and after treatment with nucleophiles are expressed as per cent of activity of the enzyme before addition of DTNB. In some experiments, TNB enzyme free from DTNB was prepared by chromatography on a column (0.5 x 10 cm) of Bio Gel P 10. The molecular sieve was equilibrated with 0.1 mM sodium-Hepes at pH 7.2 and the TNB-enzyme was eluted from the column in the same buffer. The effect of nucleophiles with the latter preparations on the reversal of inactivation by DTNB was essentially the same as that observed in experiments without the chromatographic step.

Amino Acid Analysis—Enzyme was hydrolyzed according to Moore and Stein (8) in 6 n HCl at 110°C in sealed evacuated tubes for 16, 40, and 64 hours. Samples were analyzed with a Beckman amino acid analyzer, model 119.

Cystine was determined as cysteic acid according to Moore (9). Tryptophan was determined spectrophotometrically according to Beaven and Holiday (10).

RESULTS

Inactivation by Sulphydryl Group Modifying Agents—DPN-linked isocitrate dehydrogenase was inactivated by a number of reagents which can react with sulphydryl groups. Preincubation with the substrate manganous isocitrate before addition of the inhibitors afforded protection with all reagents studied (Table I). The reagents differed in potency as inhibitors and in the degree of protection afforded by substrate. The latter is particularly apparent with iodoacetamide where practically no protection by substrate was observed in experiments without the chromatographic step.

Results with DNPH-treated enzyme with nucleophiles at the concentration indicated at 25°C. Aliquots were removed and assayed at the same time intervals. Inactivation was expressed as apparent pseudo-first order rate constants (kapp).

Preincubation in 50 mM sodium-Hepes at pH 7.2.

Preincubation in a solution containing 50 mM Tris-HCl, 10% glycerol, and 0.2 mM EDTA at pH 7.2.

Table I

| Reagents                        | Concentration | kapp (10⁻⁴ min⁻¹) |
|--------------------------------|---------------|------------------|
|                                |               | With protector | Without protector |
| Iodoacetate                    | 50            | 29.8            | 90.0              |
|                                | 25            | 2.8             | 66.4              |
| Iodoacetamide                  | 50            | 61.6            | 69.0              |
|                                | 25            | 23.0            | 45.7              |
| p-Chloromercuribenzene-sulfonic acid | 0.05       | 14.4           | 394               |
| N-Ethylmaleimide               | 0.025         | 7.1             | 95.5              |
|                                | 0.0125        | 1.2             | 30.8              |
| 5,5'-Dithiobis(2-nitrobenzoic acid) | 6.0         | 4.8             | 39.8              |
|                                | 0.25          | 8.7             | 80.6              |

* Iodocetate dehydrogenase (0.01 mM) was incubated with sulphydryl reagents at the concentration indicated at 25°C. Aliquots were removed and assayed at the same time intervals. Inactivation was expressed as apparent pseudo-first order rate constants (kapp).

† DPNH-Iodocetate (20 mM) and MnSO₄ (2 mM) were present in the preincubation mixture.

‡ Preincubation in 50 mM sodium-Hepes at pH 7.2.

§ Preincubation in a solution containing 50 mM Tris-HCl, 10% glycerol, and 0.2 mM EDTA at pH 7.2.

FIG. 1. Inactivation of isocitrate dehydrogenase. Enzyme (0.01 mM) was preincubated with DTNB at the concentration indicated in 50 mM Tris-HCl-10% glycerol buffer at pH 7.2, 25°C. Aliquots were removed and assayed at the time intervals indicated. Inactivation was expressed as apparent pseudo-first order rate constants (kapp). The inset shows a double reciprocal plot of the inactivation rate constants versus the DTNB concentration. The intercept on the X-axis of the straight line portion of this plot is 25.0 mM⁻¹.
Fig. 2. Number and state of half-cystine residues per enzyme subunit. DTNB at the concentrations indicated was preincubated with the enzyme at 25°C under the conditions specified below. The sulfhydryl content at varying time periods of incubation was determined as described under "Methods." Precipitate formation during incubation is indicated by the arrows: turbid solutions were clarified by centrifugation before readings were taken at 412 nm for determination of the sulfhydryl content. The conditions of preincubation were as follows: Curve a, enzyme (0.003 mM) and DTNB (2.5 mM) in 0.1 M Tris-HCl and 0.2 M sodium dodecyl sulfate at pH 7.2; Curve b, enzyme (0.003 mM) and DTNB (0.25 mM) in 0.1 M sodium-Hepes and 0.2% sodium dodecyl sulfate at pH 7.2; Curve c, enzyme (0.003 mM) and DTNB (2.5 mM) in 0.05 M sodium-Hepes and 0.8 M (NH₄)₂SO₄ at pH 7.2; Curve d, enzyme (0.0026 mM) and DTNB (0.23 mM) in 0.05 M sodium-Hepes at pH 7.2; Curve e, enzyme (0.015 mM) and DTNB (0.25 mM) in 5 mM sodium phosphate and 0.8 M (NH₄)₂SO₄ at pH 7.2.

**Table II**

| Amino acid composition of DPN-linked isocitrate dehydrogenase |
|---------------------------------------------------------------|
| **Amino acid** | **No. of residues per subunit** |
|----------------|-------------------------------|
| Lysine         | 25.6                          |
| Histidine      | 11.9                          |
| Arginine       | 17.1                          |
| Aspartic acid  | 42.3                          |
| Threonine      | 21.8                          |
| Serine         | 19.4                          |
| Glutamic acid  | 38.7                          |
| Proline        | 17.1                          |
| Glycine        | 32.3                          |
| Alanine        | 36.0                          |
| Valine         | 30.9                          |
| Methionine     | 13.0                          |
| Isoleucine     | 26.0                          |
| Leucine        | 22.9                          |
| Tyrosine       | 9.3                           |
| Phenylalanine  | 12.2                          |
| Half-cystine*  | 6.0                           |
| Tryptophan*    | 2.2                           |

* Values from extrapolation (8).
* Determined as cysteic acid (9).
* Determined spectrophotometrically (10).

only approximately three sulfhydryl groups per subunit were modified after 80 min (Fig. 2, Curve e).

A pseudo-first order plot of the data from an experiment (Fig. 2, Curve d) in which the mixture was preincubated with a relatively high concentration of DTNB in 0.1 M sodium-Hepes at pH 7.2 is shown in Fig. 2 (inset). The biphasic nature of this plot indicates a difference in the reactivities of the 6 sulfhydryl residues, groups modified in the initial time period being more reactive than those modified later. The intercept of the lines occurs at 38 min (Fig. 2, inset), a point indicating modification of 3.4 thiol groups per subunit (Fig. 2, Curve d).

**Inactivation and Modification**—Incubation of the enzyme with a moderate excess of DTNB ([DTNB]/[subunit] = 0.6) in 0.1 M sodium-Hepes buffer at pH 7.0 resulted in progressive modification and inactivation of the enzyme with time (Fig. 3A). A linear correlation between enzyme inactivation and modification by DTNB was observed when up to 2.8 sulfhydryl groups had been modified and 70% of the activity had been lost (Fig. 3A, inset). Interpolation of the line in Fig. 3A (inset) complete inactivation indicates that loss of activity is correlated with the modification by DTNB of three to four sulfhydryl groups on the enzyme. A similar relationship between number of sulfhydryl groups modified and inactivation was observed in the reaction of the enzyme with N-[(4-chloro)methyl]maleimide.

Changes in the composition of the preincubation mixture altered the pattern of inactivation and modification of the enzyme by DTNB (Fig. 3, B and C). In the presence of buffer containing 0.8 M (NH₄)₂SO₄ and a small excess of reagent ([DTNB]/[subunit] = 5) about 1 sulfhydryl group per subunit was modified in 60 min; however, there was essentially no change in enzyme activity during this period (Fig. 3B). The addition of a second and larger amount of DTNB ([DTNB]/[subunit] = 50) at the end of the 60-min incubation period resulted in loss of enzyme activity with time, almost complete inactivation occurring when two additional sulfhydryl groups per subunit had been modified (Fig. 3B). Even though manganous isoascorbate protected against inactivation, reaction of the enzyme with DTNB ([DTNB]/[subunit] = 21) occurred. Under these conditions, approximately two sulfhydryl groups could be modified with retention of 90% of the catalytic activity (Fig. 3C).

**Protection against Inactivation**—The effects of substrates and cofactors on rates of inactivation by two levels of DTNB in a buffer containing 50 mM Tris-HCl, 10% glycerol, and 0.2 mM EDTA at pH 7.2 is shown in Table III. The combination of Mn²⁺ and isocitrate gave the most effective protection as noted with other modifying agents (Table I); equivalent concentrations of total Mn²⁺ or total Mn⁺ were ineffective when added singly. Some protection was afforded by 0.2 mM DPNH and by DPN⁺ at 0.5 and 5 mM. However, ADP at a concentration (0.25 mM) where it is a positive modifier of the enzyme (3), was ineffective when added singly or in combination with DPN⁺.

Manganous isocitrate has been observed to give protection against inactivation in a number of situations (Tables I and III). Under certain conditions the presence of elevated concentrations of electrolytes had a differential effect on enzyme modification and inactivation (Fig. 3B). The effect of the ionic composition of the preincubation medium was, therefore, examined in more detail in the absence and presence of manganous isocitrate, the protector against inactivation. At the concentration of DTNB used in the experiments shown in Table IV ([DTNB]/[subunit] = 25), there was relatively little protection by 0.5 M (NH₄)₂SO₄ in the absence of manganous isocitrate when compared to sodium-

2 In earlier studies on inactivation by 67 μM p-chloromercuribenzenesulfonate, where no protection against inactivation by DPN⁺ or DPNH could be detected, the inhibitor was added to the assay mixture. In the present investigations inhibitors and enzyme were preincubated for varying time periods before dilution into the assay medium. Under the latter conditions and in the presence of low concentrations of p-chloromercuribenzenesulfonate (12.5 μM) (Table I) about the same protection by DPN⁺ and DPNH against inactivation by the mercurial could be obtained as with DTNB (Table III).
Enzyme activity retained (O—O).

All reactions were carried out at 23°C. A, enzyme (0.026 mM) and DTNB (0.25 mM) in 0.1 M sodium-Hepes, pH 7.07. B, enzyme (0.01 mM) was treated with 0.05 mM DTNB at 0 min. A further amount of DTNB (0.5 mM) was added at 60 min. The incubation was done in 5 mM sodium phosphate and 0.8 M ammonium sulfate at pH 7.2. C, enzyme (0.0047 mM) and DTNB (0.1 mM) in a solution containing 14% glycerol, 50 mM sodium-Hepes, 5.3 mM DL-isocitrate, and 1.3 mM MnSO₄ at pH 7.06.

TABLE III
Effects of substrates and cofactors on inhibition by DTNB

| Protectors | DTNB | k<sub>app</sub> (10<sup>-4</sup> min)<sup>-1</sup> |
|------------|------|-------------------|
| None       | 0.4  | 112               |
| DL-isocitrate (20 mM) | 0.04 | 13.1              |
| DL-isocitrate (20 mM), Mn<sup>2+</sup> (2 mM) | 0.4 | 112               |
| DPNH (0.2 mM) | 0.4 | 73.5              |
| ADP (0.25 mM) | 0.4 | 111               |
| None       | 0.2  | 70.9              |
| DPN<sup>+</sup> (0.5 mM) | 0.2 | 58.2              |
| DPN<sup>+</sup> (0.5 mM), ADP (0.25 mM) | 0.2 | 58.2              |
| DPN<sup>+</sup> (0.5 mM), Mn<sup>2+</sup> (2 mM) | 0.2 | 49.8              |
| DPN<sup>+</sup> (5 mM) | 0.2 | 32.8              |
| DPN<sup>+</sup> (5 mM), ADP (0.25 mM) | 0.2 | 32.8              |

<sup>a</sup> Enzyme (0.01 mM) was incubated with DTNB at the concentration indicated. Preincubation was carried out in 50 mM Tris·HCl-10% glycerol buffer at pH 7.2 and 23°C. Aliquots were taken and assayed at varying time intervals. Inactivation was expressed as the pseudo-first order rate constants (k<sub>app</sub>).

<sup>b</sup> The concentrations of compounds added is shown in parentheses.

Hepes buffer alone; however, 0.8 M Li₂SO₄ decreased the rate of inactivation appreciably.

Protection by manganese isocitrate against inactivation by DTNB was influenced markedly by the ionic composition of the incubation medium (Table IV). The protection was more pronounced in 50 mM Tris·HCl-10% glycerol buffer than in 50 mM sodium-Hepes buffer. The protection declined upon addition of (NH₄)₂SO₄ or Li₂SO₄ to the Hepes buffer media, and variations in salt concentration had a greater effect on protection against inactivation by manganese isocitrate than on the rate of inactivation by DTNB.

Enzyme inactivation was not affected by manganese isocitrate in preincubation mixtures containing 0.8 M Li₂SO₄. The differences in concentrations of (NH₄)₂SO₄ and Li₂SO₄ used in experiments shown in Table IV do not permit a direct comparison; however, separate experiments have shown lithium salts to be more effective than sodium or ammonium salts in slowing the interaction of enzyme with a number of group specific reagents. This may indicate relatively specific binding of Li<sup>+</sup> to a cationic site(s) located near a sulphydryl group associated with enzyme activity.

Effect of Temperature on Inactivation—Inhibition of activity
subunit had been modified. An almost complete lack of reversibility was observed in separate experiments where less than two sulfhydryl groups per subunit had been modified. Dithiothreitol was more effective than 2-mercaptoethanol; with enzyme in which three sulfhydryl groups had been modified, 5 mM dithiothreitol led to essentially complete reversal of inhibition when only a 39% reduction of disulfide bonds the reagent favors formation of a mixed disulfide. This is consistent with the previous observation that in the human liver DTNBS isocitrate dehydrogenase modified initially in different degrees by DTNB are shown in Table VI. Complete regeneration of activity was obtained with 70 mM 2-mercaptoethanol. The effectiveness of reduction of disulfide linkages (12, 13).

Modification of more than three sulfhydryl groups per subunit led to irreversible denaturation of the enzyme as indicated by protein precipitation and the observation that only a 39% reversal of inhibition was obtained with 2-mercaptoethanol when 3.5 sulfhydryl groups per subunit had been modified. An almost complete lack of reversibility was observed in separate experiments when more than four sulfhydryl groups per enzyme subunit had been modified.

Addition of sulfite to DTNB modified enzyme led to quantitative liberation of TNBS and, presumably, formation of the S-sulfo analogue of the enzyme. However, this reaction was not accompanied by restoration of activity (Table VI) indicating that the product of sulfitolysis is enzymatically inactive. The effect of sulfite is not an inactivation of the enzyme per se since treatment of the DTNB-modified enzyme preparations did not lead to a decrease in activity (Table VI) and reaction of native isocitrate dehydrogenase with the reagent did not result in inactivation.

It was shown in separate experiments that reaction of cyanide with DTNB modified enzyme led to stoichiometric liberation of TNBS and corresponding formation of the enzyme thiocyanate compound as determined by the method of Schneider and Westley (16). Cyanolysis gave 20 to 30% reversal of inhibition when two to 3.6 sulfhydryl groups had been modified by DTNB; however, a much smaller effect was observed when 1.5 groups had been modified mutually. Replacement of the sulfhydryl group at or near the active center by thiocyanate with retention of activity has been reported for aspartic transcarbamylase (13) and TPN-linked isocitrate dehydrogenase from Azotobacter vime landii (17). However, it is doubtful whether this completely explains cyanide reversal of DTNB-inactivation in DPN-linked isocitrate dehydrogenase from bovine heart.

### TABLE V

| Temperature | [DTNB] | k_{app} | Ratio |
|-------------|--------|---------|-------|
|             |        | With protector | Without protector |       |
| 23°         | 0.25   | 20.7     | 91.4   | 0.26 |
| 0           | 0.0025 | 14.3     | 24.5   | 0.57 |
| 0           | 0.25   | 35.8     | 43.5   | 0.83 |
| 0           | 0.125  | 28.0     | 32.4   | 0.86 |

* Isocitrate dehydrogenase (0.01 mM) was incubated with DTNB in 50 mM sodium-Hepes at pH 7.2. Temperature and DTNB concentrations were varied as indicated. Inactivation was expressed as apparent pseudo-first order rate constants (k_{app}).

### TABLE VI

Reversal of DTNB caused inactivation by various nucleophiles

| Nucleophile | Before treatment | After treatment | Reactivation | Third group modified |
|-------------|----------------|----------------|--------------|---------------------|
|            | (A)            | (B)            |              | number/subunit      |
| 2 Mercaptoethanol (70 mM) | 7 | 3 | 30 | 3.5 |
| KCN (5 mM) | 9 | 28 | 21 | 3.6 |
| Na$_2$SO$_4$ (5 mM) | 28 | 31 | 5 | 2.3 |
| Dithiothreitol (5 mM) | 18 | 95 | 94 | 3.1 |

* Enzyme was preincubated at 23° with DTNB in 5 mM sodium phosphate and 0.8 mM (NH$_4$)$_2$SO$_4$ at pH 7.2. The modification of sulfhydryl groups was terminated by the addition of appropriate nucleophiles. The extent of modification and enzyme activities before and after the addition of nucleophiles was determined as described under "Methods." The preincubation conditions were as follows.

| a | 0.000 mm enzyme and 0.05 mm DTNB. |
| b | 0.018 mm enzyme and 0.25 mm DTNB. |
| c | 0.000 mm enzyme and 0.25 mm DTNB. |
| d | 0.000 mm enzyme and 0.05 mm DTNB. |
| e | 0.014 mm enzyme and 0.30 mm DTNB. |
| f | 0.007 mm enzyme and 0.25 mm DTNB. |

* Unpublished results.
isocitrate, two sulfhydryl groups can be modified by DTNB

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dry1 groups per subunit had been modified (Table VI) suggests
that modification by DTNB does not destroy the protein struc-

0.2 mM DTNB (O--O) or 0.1 mM DTNB (A--A) in 25 mM
of the three more reactive sulfhydryl groups on the enzyme (Fig
the original activity could be restored by thiol containing com-

the concentration of DTNB was raised (Fig. 2, Curve c) or when
and it is likely that the 3 less reactive residues are buried inside
in the presence of protein denaturants (Fig. 2, Curves a and b),
partially derivatized enzyme.

The pH rate profile of enzyme inactivation was examined between pH 6.0 and pH 8.4. The reaction mixtures
were preincubated in a buffer containing 25 mM sodium-Hepes
and 0.5 mM (NH4)2SO4 at two concentrations of inhibitor
([DTNB] : [enzyme subunit] = 10 and 29). The plots of pseudo-
first order rate constants of inactivation versus pH shown in
Fig 4 could be fitted to sigmoidal curves and the inflection points
were calculated from a polynomial equation which gave the best
fit to these curves. The rates of inactivation differed at the
two levels of DTNB used; however, identical values of pKs
7.6 were obtained with both concentrations of inhibitor.

**DISCUSSION**

The 6 cysteine residues per subunit of DPN-linked isocitrate
dehydrogenase can be divided into two groups differing in reac-
tivity with DTNB. Under mild conditions only three sulfhy-
dryl groups were modified (Fig. 2, Curve e). However, the re-
mainng residues reacted with prolonged incubation time when
the concentration of DTNB was raised (Fig. 2, Curve c) or when
the ionic strength of the buffer was lowered (Fig. 2, Curve d and
inset). All six sulfhydryl groups are easily accessible to DTNB
in the presence of protein denaturants (Fig. 2, Curves a and b),
and it is likely that the 3 less reactive residues are buried inside
of the protein matrix and have a role in maintaining the struc-
ture of the protein. The possibility that modification of these
residues leads to unfolding of the polypeptide chains and destruc-
tion of the proper protein conformation is supported by the ob-
servations that reaction of more than 3 sulfhydryl groups with
DTNB led to protein precipitation and that less than 10% of
the original activity could be restored by thiol containing com-
pounds after more than four sulfhydryl groups per subunit of
isocitrate dehydrogenase had been modified (cf. Table VI).

Loss of activity can be correlated with modification by DTNB
of the three more reactive sulfhydryl groups on the enzyme (Fig
3A, inset). The extensive reversal of inactivation by 2-mer-
 captoethanol and dithiothreitol when fewer than three sulfhy-
dryl groups per subunit had been modified (Table VI) suggests
that modification by DTNB does not destroy the protein structure
irreversibly. However, only one of the three reactive cyste-
ine residues appears to be related to activity since under selec-
tive conditions and in the presence of the substrate manganous
isocitrate, two sulfhydryl groups can be modified by DTNB
with almost complete retention of activity (Fig. 3C). The
relative lack of conformational distortion of enzyme in which
two sulfhydryl groups per subunit have been modified is also
supported by the observation that the rate of inactivation by
DTNB in the absence or presence of protector is identical for
such a TNB-enzyme derivative and the native enzyme (Table
IV). The location of the single activity-related cysteine residue
at or near the active center of the enzyme is favored by the ob-
servation that manganous isocitrate and, to a lesser extent,
DPN+ and DPNH protect the enzyme against inactivation by
DTNB (Table III). Nevertheless, it is not completely certain
whether this thiol group per se is necessary for catalysis since
partial restoration of activity was obtained upon cyanolysis of
DTNB-inactivated enzyme in which more than two thiol groups
had been modified (Table VI). However, the fact that cyanide
brought about practically no reversal of inactivation when 1.5
residues of the enzyme had been modified (Table VI) suggests
that conversion of a sulfhydryl to a thiocyanate group at the
catalytic center leads to loss of activity. It was shown in sepa-
rate experiments with enzyme preparations at the lower level of
inactivation, freed from unreacted DTNB by gel filtration, that
there is a displacement by cyanide of TNB accompanied by a
stoichiometric appearance of thiocyanate groups on the protein
as determined by the method of Schneider and Westley (16).
The partial restoration of activity of enzyme preparations in
which 2 to 3.6 thiol groups had been modified (Table VI) may
be related to the divergence from linearity of enzyme modifica-
tion and inactivation when more than 70% of the activity has
been lost (Fig. 3A, inset). It may be that subsequent to the
initial reaction with DTNB of 1 to 2 sulfhydryl residues there
is increased modification of groups, other than the activity-
related cysteine residue, which are involved in maintaining the
conformation of the enzyme essential for activity. Replacement
of the bulky TNB substituents by the smaller thiocyanate groups
at the latter cysteine residues of the protein may lead to release
of steric changes or conformational strains which allow a partial
restoration of activity in enzyme molecules in which unmodified
activity-related sulfhydryl groups are retained. The situation
is further complicated by the fact that active DPN-linked iso-
citrate dehydrogenase contains eight apparently identical subunits
(4). It is presently unknown whether recombination of subunits
modified to different degrees can lead to formation of active
partially derivatized enzyme.

The pH rate profile of enzyme inactivation by DTNB suggests
that the pKs of the active center is at 7.6 and that the reactive
species is a mercaptide ion (Fig. 4). This value of pKs is lower
by 2.6 pH units than might be expected for a cysteine residue of
a protein (18, 19). The relatively low pKs values of the activity-
related sulfhydryl group and of a specific amino group described
previously (5, 6) suggest that these residues of DPN-linked iso-
citrate dehydrogenase are located in a hydrophobic environment.
Support for the presence of a hydrophobic region in DPN-linked
isocitrate dehydrogenase which is related to enzyme activity
has been obtained in recent studies with fluorescent probes.4
The influence of a hydrophobic environment on the reactivity of
thiol groups has been demonstrated in studies of a series of N-acyl
cysteine derivatives in micelles (20). The values predicted for
the free energy change for formation of thiol hydrophobic bonds
in this model system agreed with those calculated previously by
Nemethy and Scheraga (21) and Tanford (22).

The effects of changes in electrolyte concentration and tem-
perature give further insights into the reactivity of the enzyme

4 C. C. Fan, L. Tomcho, and G. W. E. Plaut, unpublished ob-
observations, 1973.
with DTNB and substrate. The rate of inactivation by 0.25 mM DTNB declined only moderately when the electrolyte concentration of the medium was raised (Table IV); nevertheless, in the presence of 0.05 mM DTNB and 0.8 mM (NH₄)₂SO₄, one sulfhydryl group per enzyme subunit could be modified without affecting activity (Fig. 3B). The seemingly contradictory observations may be due to differences in environment in which the different sulfhydryl groups are located on the enzyme. Thus, one would expect with increasing electrolyte concentration a lower reactivity of mercaptide ion as predicted from the primary salt effect (23) and, at the same time, a decrease in charge interaction with neighboring groups which should promote the disulfide interchange reaction with DTNB. If these opposing effects are about equal, in the case of the activity-related sulfhydryl group of isocitrate dehydrogenase, the rate of inactivation would tend to be influenced little by changes in salt concentration. Such effects also have been observed in the reactions of DTNB with ficin and creatine kinase (24). The rapid modification of a reactive amino group located at or near the catalytic center of the enzyme over the temperature range examined which might be consistent with the observed cold inactivation of DPN-linked isocitrate dehydrogenase from liver (2). This may be accompanied by a decrease in affinity or by a decreased shielding by bound manganous ions which increase the reactivity of this group with DTNB or both.

Variations in salt concentration led to a relatively larger effect on protection against inactivation by the substrate manganous isocitrate than on the rate of inactivation by DTNB (Table IV). This would be consistent with the suggestion that the active center sulfhydryl group is an environment which is not highly charged and is hydrophobic. However, the binding of the substrate seems to be strongly associated with the charge of another functional group(s) on the enzyme. The latter may be due to a reactive amino group located at or near the catalytic center of the enzyme which shows a markedly decreased rate of reaction with a number of amino group specific reagents with increasing ionic strength (5, 6).

The changes in the rate of inactivation and protection by manganous isocitrate by lowering the temperature are qualitatively similar to those observed with increasing salt concentrations. There is about a 50% decrease in inactivation by 0.25 mM DTNB in the absence of protector when the temperature is lowered from 23 to 0°C. This could be attributed to the dependence of reaction rate on temperature and to the increase in the pKₐ of the essential sulfhydryl group at lower temperature which could be predicted from the heat of ionization of this group (19). Under the same conditions there is almost no protection by substrate at 0°C. Indeed, in the presence of protector the rate of inactivation by DTNB is higher at 0 than at 23°C (Table V). These results could suggest a conformational change of the protein over the temperature range examined which might be consistent with the observed cold inactivation of DPN-linked isocitrate dehydrogenase from liver (2). This may be accompanied at the lower temperature by a decline of enzyme-substrate binding affinity or by a decreased shielding by bound manganous isocitrate of the reactive sulphydryl group, due to a greater geometric separation of functional groups at or near the catalytic center of the enzyme.

There appear to be at least 2 functional amino acid residues at the active center of DPN-linked isocitrate dehydrogenase of bovine heart. There is an essential lysine residue primarily involved in the binding of manganous isocitrate to the enzyme. The substrate protects the enzyme and causes reversal of inhibition by pyridoxal phosphate, while DPN⁺ and DPNH are ineffective in this respect (5, 6). The activity-related sulfhydryl group studied here seems to be located between binding sites for the substrate and the pyridine nucleotides since manganous isocitrate, DPN⁺, and DPNH protect the enzyme against inactivation by DTNB (Table III). It has been found recently that DPN-linked isocitrate can catalyze the hydrolysis of p-nitrophenylacetate, which inhibits the dehydrogenase activity; the esterase activity can be inhibited by DTNB, manganous isocitrate, DPN⁺, ADP, or DPNH. These observations support the location of a cysteine residue at the active center of the enzyme. However, it is uncertain whether this sulfhydryl group participates directly in catalysis, perhaps as a mediator of the transport of hydride ion from substrate to DPN⁺ by a type of mechanism proposed by Wang (25) for alcohol dehydrogenase, or whether it functions in maintaining enzyme conformation required for activity.

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