the concentration of free $K^+$ and $Mg^{2+}$ must be low enough to prevent protection. How other cell constituents effect the rate of inactivation at 23° is also not known.

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# Oxidized Triphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase from *Azotobacter vinelandii*. Modification of a Reactive Sulfhydryl Group with Cyanide*

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ABSTRACT: A thiocyano derivative of *Azotobacter vinelandii* isocitrate dehydrogenase has been prepared. The modified enzyme is prepared by displacing a thionitrobenzoate group from monothionitrobenzoate derivative of the enzyme with cyanide. The replacement of the thionitrobenzoate group by cyanide results in the regeneration of catalytic activity in the enzyme. The results indicate that the most reactive thiol group of the enzyme is not essential for catalytic activity although its modification by iodoacetic acid, *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, and 5,5′-dithiobis(2-nitrobenzoic acid) results in almost complete loss of catalytic activity. The Michaelis constants for both $TPN^+$ and isocitrate were unchanged by the modification of the native enzyme. The pH stability of the thiocyano derivative was determined, the enzyme is stable at pH 8 and 9.2 but becomes increasingly unstable at lower pH values. In the thiocyano enzyme a second thiol residue previously inaccessible to Ellman's reagent becomes accessible for titration.

The $TPN^+$-specific isocitrate dehydrogenase from *Azotobacter vinelandii* (ATCC 9104) contains three cysteine residues which exhibit different reactivities toward thiol reagents (Chung and Franzen, 1969). One of the thiol groups of the enzyme is readily titrated with DTNB.[1] This titration results in an almost complete loss of catalytic activity. The enzymatic activity is restored by treatment of the modified enzyme with DTT; this restoration of activity is accompanied by a stoichiometric release of the TNB chromophore and regeneration of the free thiol group of the enzyme. The reactive thiol group is also titrated by iodoacetic acid with complete loss of catalytic activity. Concomitantly one carboxymethyl group is bound per mole of enzyme. Treatment of the enzyme with HMB results in the rapid titration of two thiol groups with complete loss of catalytic activity and the slow titration of the third thiol group. The proximity of the most reactive thiol to the active site of the enzyme is indicated by the observation that substrates of the enzyme decrease the reactivity of the thiol with respect to DTNB. The thiol groups of the enzyme exist in an interesting steric relationship. Titration of the reactive thiol with DTNB and subsequent exposure of the mono-TNB derivative of the enzyme to mild denaturing conditions results in the formation of an enzyme species which contains an

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; HMB, *p*-hydroxymercuribenzoate; TNB, thionitrobenzoate; IDH, isocitrate dehydrogenase.

intramolecular disulfide bridge (Braginski *et al.*, 1970). In the formation of this modified form of the enzyme the bound chromophore is displaced. The disulfide enzyme is devoid of activity but upon reduction with DTT partial restoration of activity is observed. The proximity of the reactive thiol group to the active center of the enzyme and the interesting steric relationship and differential reactivities of the three thiol residues prompted a further investigation into the functional roles of these residues in the enzyme. In this communication the preparation and some general properties of a thiocyanoalanine derivative of the enzyme are described.

## Experimental Section

*Materials.* All nucleotides and coenzymes, *threo*-$D_sL_s$-isocitrate trisodium salt, DTNB, DTT, and HMB were purchased from Sigma. [$^{14}$C]Potassium cyanide was obtained from New England Nuclear, Boston, Mass. All other reagents were analytical grade obtained from various commercial sources.

*Preparation of Isocitrate Dehydrogenase.* Isocitrate dehydrogenase with a specific activity of 110–130 enzyme units/mg of protein was prepared by a modification of the method described by Chung and Franzen (1969). In the modified method the CM-cellulose fractionation was omitted and the final SephadexG-100 chromatography repeated. This modified method resulted in a higher yield of enzyme which was of comparable purity as determined by acrylamide gel electrophoresis (Ornstein, 1964; Davis, 1964), and sedimentation velocity analysis in the Spinco Model E analytical ultracentrifuge.

*Preparation of TNB–IDH.* The monothionitrobenzoate derivative of isocitrate dehydrogenase (TNB–IDH) was prepared by titrating isocitrate dehydrogenase with Ellman's reagent (Ellman, 1959) until one thiol group had been titrated and less than 5% of catalytic activity remained. A typical preparation was carried out as follows. Enzyme (90 mg) was dissolved in 44 ml of 0.05 M potassium phosphate buffer (pH 7.0) and mixed with 0.5 ml of a solution containing DTNB at a concentration of 4 mg/ml of 0.05 M potassium phosphate buffer (pH 7.0). The solution was allowed to stand in the dark at room temperature. The course of the reaction was monitored by measuring the absorbance of the solution at 412 nm at fixed intervals. The reaction was complete within about 30 min at which time approximately 1 equiv of thiol/mole of enzyme was titrated. This preparation contained less than 5% of the activity of the native enzyme. Complete activity could be regenerated by the addition of excess DTT. The solution containing the titrated enzyme was freed of excess DTNB and released TNB chromophore by gel filtration on a Sephadex G-25 column. The protein solution obtained after concentration by ultrafiltration was used for the preparation of the thiocyanoalanyl derivative of isocitrate dehydrogenase (SCN–IDH).

*Preparation of SCN–IDH.* The thiocyanoalanyl derivative of the enzyme was prepared as follows. In a standard spectrophotometer cuvet 12 mg of TNB–IDH in 2 ml of 0.13 M potassium phosphate buffer (pH 7.2) was mixed with 1 ml of a freshly prepared solution containing 9.1 mg of potassium cyanide/ml of 0.5 M postassium phosphate buffer (pH 7.2). The reaction mixture was immediately placed into the cell compartment (maintained at 30°) of a Cary Model 1605 recording spectrophotometer and the release of the TNB chromophore followed at 412 nm. The release of chromophore was complete after about 25 min. The net change in absorbance was calculated from the final reading and the absorbance of the initial enzyme solution after correction for dilution by the potassium cyanide solution. In the preparation of the radioactive derivative the procedure was unchanged except that [$^{14}$C]potassium cyanide replaced the unlabeled compound.

*Enzyme Assay and Kinetics of Enzyme Reaction.* The enzyme activity was measured as previously described (Chung and Franzen, 1969). The kinetic constants for $TPN^+$ and isocitrate were obtained in the usual manner (Cleland, 1970). The effect of varying the isocitrate concentration at several fixed nonsaturating concentrations of $TPN^+$ on the rate of reaction was determined. Primary plots of reciprocal initial velocity *vs.* reciprocal varying substrate concentrations were obtained, from these plots secondary plots of intercepts against the reciprocal of fixed substrate concentrations were made. These secondary plots yielded the Michaelis constants for $TPN^+$ and isocitrate.

*Protein Determination.* Protein concentration was determined either by the absorption of the protein solution at 280 nm (Chung and Franzen, 1969) or by the method of Lowry *et al.* (1951) with native IDH as standard.

## Results

*Stoichiometry of Reaction of TNB–IDH with [$^{14}$C]KCN.* The TNB–IDH which was treated with [$^{14}$C]KCN contained 0.9 mole of TNB/mole of enzyme as determined by release of chromophore after treatment with excess dithiothreitol. The release of chromophore by treatment with [$^{14}$C]KCN was 0.74 mole/mole of enzyme. Measurement of the radioactivity bound to the enzyme after exhaustive dialysis indicated that 1.18 moles of cyanide was bound/mole of enzyme. Incubation of the radioactive enzyme with sodium hydroxide at pH 12 released thiocyanate, which was assayed by the method of Sorbo (1957). The release of thiocyanate was 1.06 moles of thiocyanate/mole of enzyme. These data are summarized in Table I and indicate that cyanide reacts with TNB—IDH, releases approximately 1 mole of chromophore/mole of enzyme, and forms 1 mole of thiocyano derivative which is presumed to be thiocyanoalanine in analogy with model reactions (Wood and Catsimpoolas, 1963; Catsimpoolas and Wood, 1966) and the formation of thiocyanoalanyl derivatives in the regulatory subunit of aspartate transcarbamylase (Vanaman and Stark, 1970).

*Kinetics of Thionitrobenzoate Release from TNB–IDH by $CN^-$.* The kinetics of release of chromophore under the standard experimental conditions previously described were studied. The log of the final absorbance at 412 nm minus the absorbance at time, $t$, was plotted against time. From the slope of the plot the first order-rate constant determined for the displacement of the TNB from TNB–IDH was 0.17 $min^{-1}$. The $t_{1/2}$ for the reaction was 4.1 min.

*Properties of the SCN–IDH.* The SCN–IDH yielded one symmetrical peak in the analytical ultracentrifuge. This peak had a sedimentation constant of 4.9 S. Analysis by Sephadex G-100 chromatography of the radioactive SCN–IDH yielded one radioactive peak which was coincident with the catalytic activity of the enzyme. The elution volume was identical with that of an untreated homogeneous preparation of IDH. The modified enzyme was inactivated by rabbit antibody prepared against native enzyme and yielded one precipitin band by the double-diffusion Ouchterlony test against the antibody to native enzyme. These results indicate that the SCN–IDH retained its monomeric structure and its native conformation.

The samples of SCN–IDH were catalytically active and had specific activities which varied between 40 and 70 enzyme

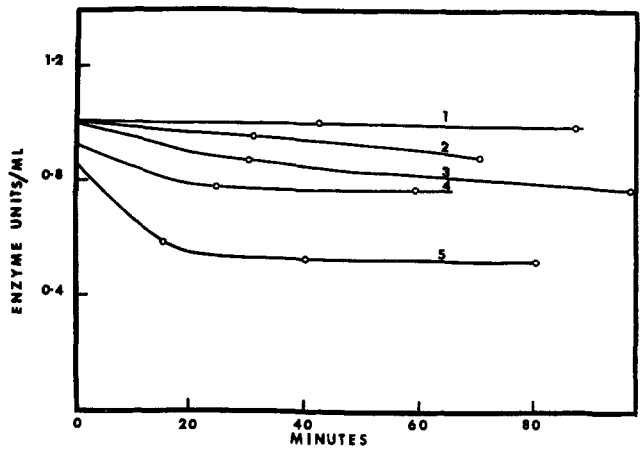


FIGURE 1: pH stability of SCN–IDH. SCN–IDH was diluted in various buffers to yield a final protein concentration of 18 μg/ml. Prior to exposure to the various buffers the specific activity of the enzyme was 64 enzyme units/mg of protein. Immediately after dilution in the appropriate buffer an aliquot of the diluted enzyme solution was assayed for activity. The solution was allowed to incubate at 24° and at fixed time intervals aliquots were assayed for activity. The buffers used were: pH 3.9, 0.1 M sodium acetate; pH 5.0, 0.05 M sodium acetate; pH 5.8, 0.05 M potassium phosphate; pH 7.0, 0.05 M potassium phosphate; pH 8.2, 0.5 M Tris-acetate; pH 9.1, 0.05 M Tris-acetate. The enzyme was almost instantaneously inactivated at pH 3.9 and its change in activity is not recorded. The pH of the incubation for curves 1–5 are 9.1, 8.2, 7.0, 5.8 and 5.0, respectively.

units/mg of protein. The TNB–IDH which was normally used to prepare the SCN–IDH had specific activities of less than 5 enzyme units/mg of protein. These specific activities may be compared to that of native enzyme which varied between 110 and 130 enzyme units per mg of protein.

The kinetics of the reduction of $TPN^+$ by isocitrate catalyzed by the SCN–IDH was studied at several concentrations of $TPN^+$ and isocitrate. The plots of reciprocal velocities against reciprocal substrate concentrations where isocitrate and $TPN^+$ were the variable substrates, respectively, were linear. Linear secondary plots of intercept *vs.* the reciprocal of fixed substrate concentrations were obtained from these plots. Analysis of these secondary plots yielded Michaelis constants for isocitrate and $TPN^+$ of $8.2 \times 10^{-6}$ and $1.1 \times 10^{-5}$ M, respectively. These values may be compared with $1.1 \times 10^{-5}$ M for isocitrate and $1.0 \times 10^{-5}$ M for $TPN^+$ when unmodified enzyme was used under identical conditions of pH, temperature, ionic strength, and metal ion concentration.[2] These Michaelis constants are essentially unchanged in the modified enzyme.

The pH stability of the SCN–IDH enzyme was determined. The results are shown in Figure 1. The modified enzyme is stable at room temperature at pH values between 8 and 9 but loses approximately 40% of its activity after incubation at pH 5 for 20 min. The residual activity appears to be stable at this pH. At pH 3.9 the enzyme is almost instantaneously inactivated.

Treatment of [$^{14}$C]SCN–IDH with mercaptoethanol at room temperature for 2 hr and subsequent dialysis resulted in partial release of radioactivity. The release of radioactivity was followed by an increase in the specific activity of the enzyme and an increase in the number of titratable thiol groups. In contrast, treatment of the enzyme with 4 M guanidine hydrochloride resulted in a loss of activity and no loss of

[2] J. Wicken, unpublished data.

TABLE I: Stoichiometry of Reaction of TNB–IDH with [$^{14}$C]KCN.[a]

| Reaction | Stoichiometry (mole/mole of Enzyme) |
|---|---|
| TNB released by $CN^-$ | 0.74 |
| [$^{14}$C]$CN^-$ bound | 1.18 |
| SCN released | 1.06 |

[a] The TNB released by cyanide was determined as described in the text. After reaction of TNB–IDH with [$^{14}$C]KCN as described, the reaction mixture was exhaustively dialyzed against 0.05 M potassium phosphate buffer (pH 7.0) until the dialysate was free of radioactive cyanide. The protein content of the dialyzed enzyme solution was determined spectrophotometrically and 0.1 ml of the solution was removed and mixed with 10 ml of a solution containing 5.5 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 667 ml of toluene, and 333 ml of Triton X-100 for radioactivity measurement. The radioactivity was determined with a Beckman LS-100 liquid scintillation counter. The specific activity of the [$^{14}$C]KCN used for the reaction was $10^6$ cpm/μmole. The number of moles of [$^{14}$C]$CN^-$ bound was determined from the radioactivity in the dialyzed sample. The stoichiometry of binding was determined from this value and the protein concentration. The stoichiometry of $SCN^-$ released was determined according to Sorbo (1957). SCN–IDH (5.8 mg) was incubated for 30 min at pH 12 at 37° and at the end of this time the $SCN^-$ released was determined. The color development was compared with a standard curve obtained with potassium thiocyanate.

radioactivity from the protein. However, the guanidine-treated enzyme had lost titratable thiol groups. These results are summarized in Table II. The loss in titratable thiol groups by guanidine hydrochloride treatment was perhaps due to the formation of an intramolecular disulfide bridge since the sedimentation velocity pattern of the guanidine hydrochloride treated enzyme after removal of the denaturing agent exhibited one major peak which had an $s^0_{20,w}$ of 4.1 S, this value is close to that for the native enzyme, *viz.*, 4.6 S. Furthermore, a mixture of the guanidine hydrochloride treated enzyme and native unlabeled enzyme when mixed together and chromatographed on a column of Sephadex G-100 resulted in a coincidence of radioactivity and enzyme activity in the eluted fractions. Polymerization by the formation of intermolecular disulfide bridges is thus ruled out.

It has previously been reported that only one thiol group per mole of native enzyme is readily titrated by Ellman's reagent (Chung and Franzen, 1969). This titration was usually complete within 20 min under standard conditions. No further reaction was observed. The SCN–IDH was not readily titrated by Ellman's reagent. However, exposure of the modified enzyme to Ellman's reagent overnight resulted in the further titration of a second thiol group. The progress of this reaction was followed by the release of chromophore at 412 nm on a Cary 1605 recording spectrophotometer. After removal of excess Ellman's reagent and released chromophore the enzyme was treated with potassium cyanide. This resulted in the release of bound chromophore and partial regeneration of catalytic activity. These results are summarized in Table III.

TABLE II: Effects of Exposure of [$^{14}$C]SCN–IDH to Mercaptoethanol or Guanidine Hydrochloride.[a]

| Treatment | Radioactivity Remaining (cpm/mg of Protein) | Sp Act. (Enzyme Units/mg) | Sulfhydryl Content (moles/mole of Enzyme) |
|---|---|---|---|
| None | 5130 | 48 | 1.5 |
| 0.1 M Mercaptoethanol | 2750 | 72 | 2.6 |
| 4 M Guanidine hydrochloride | 5605 | 8 | 0.5 |

[a] [$^{14}$C]SCN–IDH samples, each containing 4.3 mg of protein, were separately incubated with 0.05 M potassium phosphate buffer, or buffer containing either 0.1 M mercaptoethanol or 4 M guanidine hydrochloride for 1 hr at 23°. At the end of this time each sample which was in a total volume of 2 ml was dialyzed at 4° against three changes of 1000 ml of 0.05 M potassium phosphate buffer (pH 7.0) over a period of 24 hr. The protein content, radioactivity, and enzyme activity in aliquots of each sample were determined. The sulfhydryl content of the samples were then determined by titration with HMB by the method of Boyer (1954).

TABLE III: Modification by IDH by Ellman's Reagent and Cyanide.[a]

| Treatment | TNB Bound to Enzyme (mole/mole of Enzyme) | TNB Released from Enzyme (mole/mole of Enzyme) | Sp Act. (eu/mg of Protein) |
|---|---|---|---|
| None | | | 115 |
| DTNB | 0.9 | | 1.7 |
| DTNB + $CN^-$ | | 0.8 | 40 |
| DTNB + $CN^-$ + DTNB | 0.69 | | 8.1 |
| DTNB + $CN^-$ + DTNB + $CN^-$ | | 0.7 | 26.7 |

[a] IDH (3 ml) containing 2.9 mg of protein/ml was titrated with Ellman's reagent at room temperature in the dark. After 120 min excess reagent and released TNB were removed by gel filtration on a Sephadex G-25 column. The protein solution in 0.05 M potassium phosphate buffer (pH 7.0) was concentrated by ultrafiltration through an Amicon Diaflo apparatus. After concentration the enzyme was treated with potassium cyanide essentially as described in the text. The release of TNB from the enzyme by cyanide was determined at 412 nm in a Zeiss PMZ II spectrophotometer. The mixture was passed through a Sephadex G-25 column to remove excess reagents and released chromophore and the protein reconcentrated as before. The activity and protein content of the solutions were determined. The SCN–IDH was titrated with Ellman's reagent overnight at 23° in a Cary 1605 recording spectrophotometer. From the net change in absorbance at 412 nm and the protein concentration determined by its absorbance at 280 nm the stoichiometry of the titration of thiol groups was determined. This sample was dialyzed overnight against 0.05 M potassium phosphate buffer (pH 7.0) and its catalytic activity and protein content determined. This sample was treated once more with potassium cyanide and the release of chromophore determined at 412 nm. The release of chromophore was accompanied by a partial regeneration of catalytic activity as shown in the last line of the table.

A sample of [$^{14}$C]SCN–IDH was subjected to tryptic digestion for five hours at pH 7, 37° at an enzyme to trypsin ratio of 20:1. The resulting peptides were subjected to high-voltage electrophoresis in a Camag HVE system for 60 min at pH 3.6 with a potential gradient of 65 V/cm. The electrophoretogram was scanned for radioactivity with a Packard radiochromatogram scanner. The electrophoretogram revealed a single radioactive peak which migrated approximately 2 cm toward the anode. This result suggests that the radioactive cyanide resides on a single peptide and a single thiol residue is modified by cyanide.

## Discussion

The results reported in this communication have indicated that isocitrate dehydrogenase can be modified by a combination of treatment with Ellman's reagent and cyanide to yield a stable modified but active enzyme species which presumably has a reactive cysteine residue replaced by a thiocyanoalanyl residue. Wood and Catsimpoolas (1963) and Catsimpoolas and Wood (1966) have shown that cyanide is capable of cleaving the disulfide bonds in cystine peptides to yield a thiol and a thiocyanoalanyl derivative which subsequently cyclizes to form the *N*-acyl-2-iminothiazolidine derivative followed by hydrolysis of the *N*-acyl bond. More recently Vanaman and Stark (1970) have demonstrated the formation of a thiocyano derivative of the catalytic subunit of aspartate transcarbamylase from *Escherichia coli* by displacement of the thionitrobenzoate group with cyanide from the enzyme titrated with Ellman's reagent. In Vanaman and Stark's work it was shown that the above modification did not affect the catalytic activity of the enzyme. The thiocyano group could be released by treating the enzyme with mild alkali and could be quantitated. Their modified enzyme was stable. The experiments described here with isocitrate dehydrogenase indicate that the enzyme indeed contains one thiocyanoalanine residue by the stoichiometry of displacement of thionitrobenzoate by cyanide, the binding of radioactive cyanide in stable linkage, the loss of one thiol residue, and the release of 1 equiv of thiocyanate by mild alkali. The modified enzyme is stable and does not spontaneously release the bound radioactivity.

The bound radioactive cyanide may be partially released by treatment with 0.1 M mercaptoethanol with a regeneration of a thiol residue. Treatment of the enzyme with either 0.1 N HCl or 4 M guanidine hydrochloride did not result in the loss of radioactivity from the macromolecule. Analysis of the guanidine hydrochloride treated enzyme either by gel filtration or by analytical ultracentrifugation revealed that the enzyme did not undergo extensive fragmentation. In the model reactions previously mentioned peptide bond cleavage accompanied the cleavage of the disulfide bond and subsequent rearrangement. With the aspartate transcarbamylase subunit,

fragmentation of the protein followed denaturation. Our results indicate that if fragmentation of the SCN–IDH occurred the peptide released was not of sufficient size to alter the sedimentation or gel filtration patterns significantly, *i.e.*, if the enzyme is cleaved the modified thiol must have been close to the N terminus of the enzyme polypeptide chain. This possibility has to be explored further.

Previous work on the *Azotobacter* isocitrate dehydrogenase (Chung and Franzen, 1969) and on the pig heart enzyme (Colman, 1969; Colman and Chu, 1970) indicated that modification of thiol groups resulted in loss of catalytic activity. The thiol groups were protected to varying degrees from thiol reagents by substrates. This observation suggested that the thiol groups were involved in the catalytic function of these enzymes. Since the modifying reagents were either bulky and/or charged, other explanations are possible. The thiol modification could: (a) sterically hinder substrate binding, (b) cause a conformational change in the tertiary structure of the enzyme, or (c) alter the charge around the active site and thus modify binding of the substrates. In the experiments reported in this communication we have shown that the reactive thiol group of the *Azotobacter* enzyme is not essential for catalytic activity. Conversion of the reactive thiol of this enzyme into a thiocyano group yields an enzyme preparation which has approximately 30–50% of the catalytic activity of the native enzyme.

The inhibition of the *Azotobacter* enzyme by Ellman's reagent, HMB, and iodoacetic acetic acid may be attributed to the charge and or size of these modifying agents. It appears that the above reagents do not cause gross conformational changes in the enzyme structure since all these modified enzymes give precipitin bands with rabbit antiserum prepared against the native enzyme. Furthermore, no gross changes have been observed in their circular dichroism spectra. The thiocyano group is uncharged and less bulky than either the HMB or TNB group. This combination of lack of charge and smaller size perhaps allows substrates to bind to the active site of the thiocyano enzyme.

The kinetic studies indicate that $K_m$ for both $TPN^+$ and isocitrate were not significantly altered for the SCN–IDH when compared with native enzyme. The effect of modification then is to alter the maximal velocity of the enzyme. The manner in which this is accomplished is not clear at present. Attempts were made to determine if the SCN–IDH could react more effectively than the unmodified enzyme with $DPN^+$ and deaminoTPN$^+$ as substrates. It was found that the specificity of the modified enzyme is not much different from that of the native enzyme. The marked instability of both enzymes at pH 4 is of some interest. The instability of the SCN–IDH may possibly be due to peptide bond cleavage although this has to be established.

The accessibility of a second thiol residue of the enzyme to Ellman's reagent in the SCN–IDH is of some interest. In the native enzyme only one thiol residue can be titrated per mole of enzyme. The titration of this group apparently blocks the second thiol group from attack by the modifying reagent. Removal of the bulky charged chromophore by cyanide allows the reagent to react slowly with the second thiol group. The proximity of these two thiol groups in the native enzyme is emphasized by the following observations (a) mild denaturation of TNB–IDH results in displacement of chromophore with the generation of an intramolecular disulfide bridge (Braginski *et al.*, 1970), (b) the second thiol group in TNB–IDH is titratable if the TNB chromophore is replaced by $CN^-$.

The behavior of these two thiol groups in IDH is reminiscent of the behavior of cysteine-149 and -153 of glyceraldehyde phosphate dehydrogenase (Harris, 1970). The behavior and function of these groups may also be compared with those in the $\alpha$ subunit of tryptophan synthetase in which a very reactive sulfhydryl group protects a presumed active-site sulfhydryl group (Malkinson and Hardman, 1969). It is not clear at the moment whether or not the second thiol group in IDH is involved with the catalytic mechanism. It seems at the present time that the second thiol group can be modified in a similar manner with cyanide as the first reactive group with the retention of some catalytic activity.

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