11, 1643.
Boers, W., Oosthuizen, C., and Slater, E. C. (1971), *Biochim. Biophys. Acta 250,* 35.
Buehner, M., Ford, G. C., Moras, D., Olsen, K. W., and Rossmann (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3052.
Czeisler, J. L., and Hollis, D. P. (1973), *Biochemistry 12,* 1683.
Jardetzky, O., Wade, N. G., and Fischer, J. J. (1963), *Nature (London) 197,* 183.
Lee, C.-Y., Eichner, R. D., and Kaplan, N. O. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1593.
Pesce, A., McKay, R. H., Stolzenbach, F., Cahn, R. D., and Kaplan, N. O. (1964), *J. Biol. Chem. 239,* 1753.
Sarma, R. H., and Kaplan, N. O. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 1375.
Sarma, R. H., and Mynott, R. J. (1972), *Org. Magn. Reson. 4,* 577.
Sloan, D. L., and Mildvan, A. S. (1974), *Biochemistry 13,* 1711.
Stinson, R. A., and Gutfreund, H. (1971), *Biochem. J. 121,* 235.
Stinson, R. A., and Holbrook, J. J. (1973), *Biochem J. 131,* 719.

Coenzyme Binding by Native and Chemically Modified Pig Heart Triphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase†

Robert S. Ehrlich and Roberta F. Colman*

ABSTRACT: The binding of TPNH to native and chemically modified pig heart TPN-dependent isocitrate dehydrogenase was studied by the techniques of ultrafiltration and fluorescence enhancement. A single site (per peptide chain) was found for TPNH with a dissociation constant (K_D = 1.45 μM) that is quantitatively comparable to the Michaelis constant. The oxidized coenzyme, TPN^+, weakens the binding of TPNH. The substrate manganous isocitrate also inhibits the binding of TPNH and, reciprocally, TPNH inhibits the binding of manganous isocitrate, suggesting that binding to the reduced coenzyme and substrate sites is mutually exclusive. Ultrafiltration experiments with carbonyl [^{14}C]TPN^+ revealed the existence of two sites with a dissociation constant (49 μM) more than ten times higher than the Michaelis constant. This observation excludes a random mechanism for isocitrate dehydrogenase or a sequential mechanism in which TPN^+ binds first. Four chemically modified isocitrate dehydrogenases have been prepared: enzyme inactivated by reaction of a single methionyl residue with iodoacetate, by modification of a glutamyl residue by glycinamide (in the presence of a water soluble carbodiimide), by reaction of four cysteines successively with 5,5′-dithiobis(2-nitrobenzoic acid) and potassium cyanide, or by addition of two cysteine residues to *N*-ethylmaleimide. These enzymes were tested for their ability to bind TPN^+, TPNH, and manganous isocitrate. In the cases of the cysteinyl and glutamyl-modified enzymes, inactivation appears to be due primarily to loss of the ability to bind the substrate manganous isocitrate. In contrast, the methionyl residue may participate in the coenzyme binding site or, more likely, may be involved in a step in catalysis subsequent to binding.

The active site of pig heart TPN-dependent isocitrate dehydrogenase (*threo*-Ds-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) has been explored by means of manganese and isocitrate binding experiments (Villafranca and Colman, 1972; Colman, 1969a). These studies have shown that manganese (Villafranca and Colman, 1972) and isocitrate (Colman 1969a) bind independently but weakly to isocitrate dehydrogenase but the binding of either is substantially enhanced when both are present. Analysis of kinetic data has indicated that the preferred substrate is the manganese–tribasic isocitrate complex (Colman, 1972a). In contrast to the extensive data on the interaction of the enzyme with isocitrate and manganese, little is known of the coenzyme binding properties of isocitrate dehydrogenase. A study of the interaction of TPNH with a partially purified enzyme preparation suggested the existence of two TPNH sites with extremely tight binding (dissociation constant approximately 10^{-8} M) (Langan, 1960). Qualitative experiments indicated competition with TPNH by TPN^+ and isocitrate in the presence of manganese (Langan, 1960). In addition to its role as coenzyme in the carboxylation of α-ketoglutarate, TPNH has been shown to be necessary for the detritiation of α-ketoglutarate in the presence of isocitrate dehydrogenase (Rose, 1960). The present investigation uses a homogeneous preparation of isocitrate dehydrogenase to examine quantitatively the binding of TPNH and the interaction between TPNH, TPN^+, and manganese–isocitrate. In addition, carbonyl-labeled [^{14}C]TPN^+ has been used to measure directly the affinity of the native enzyme for TPN. Binding data for oxidized and reduced coenzymes are compared with kinetic data in order to relate them with the possible reaction mechanisms.

Information about the function of amino acid residues at the active site can be obtained by examining the characteristics of enzymes which have been chemically modified at

† From the Department of Chemistry, University of Delaware, Newark, Delaware 19711. *Received June 26, 1975.* Supported by U.S. Public Health Service Grant 5-RO1-AM 17552.

specific residues. Modified isocitrate dehydrogenase has been prepared in which a single methionyl residue has reacted with iodoacetate (Colman, 1968), an average of two cysteinyl groups have added to *N*-ethylmaleimide (Colman and Chu, 1970a,b), an average of four cysteinyl residues have reacted successively with 5,5′-dithiobis(2-nitrobenzoic acid) and cyanide (Colman, 1969b; Johanson and Colman, 1974), and a single glutamyl residue has been modified with glycinamide in the presence of water-soluble carbodiimide (Colman, 1973). The ability of these inactive, chemically altered enzymes to bind the oxidized and reduced coenzymes is examined in this study, in order to assess the contribution of these critical amino acid residues to the binding sites of isocitrate dehydrogenase.

Experimental Procedure

Materials. Pig heart TPN–isocitrate dehydrogenase was supplied by Boehringer Mannheim Corp. and purified by column chromatography on carboxymethycellulose and Sephadex G-150 as previously described (Colman, 1968). The enzyme was stored at −85° in 0.1 *M* triethanolamine chloride buffer (pH 7.7) containing 10% glycerol and 0.3 *M* sodium sulfate (standard triethanolamine buffer). Protein concentration was determined from the absorbance at 280 nm using a value of 9.1 for $E_{280}(1\%)$ (Colman, 1968) and a molecular weight of 58000 (Colman, 1972b). Isocitrate dehydrogenase activity was measured spectrophotometrically from the increase in absorbance at 340 nm using a reaction mixture containing DL-isocitrate, manganous sulfate, and TPN^+ at concentrations of 4, 2, and 0.1 m*M*, respectively (Colman, 1968).

Coenzymes, *N*-ethylmaleimide,[1] and *threo*-D_S-isocitrate were obtained from Sigma Chemical Company. Aldrich Chemical Company supplied the 5,5′-dithiobis(2-nitrobenzoic acid), glycinamide, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate.

The carbonyl-labeled [^{14}C]TPN^+ was obtained from Amersham-Searle. It was purified on a column of Whatman DE-52 ion exchange resin (1.5 cm diameter × 5.5 cm height) by the procedure of Winer (1964). The column was washed with 0.01 *M* Tris-chloride (pH 7.5) and then eluted with a linear gradient from the starting buffer (200 ml) to 0.01 *M* Tris-chloride (pH 7.5) plus 0.5 *M* NaCl (200 ml). Approximately 12% of the total counts were contained in an initial peak and about 1% in a small peak eluting before TPN^+. TPN^+ concentration was determined enzymatically using TPN–isocitrate dehydrogenase to convert it to TPNH which was then measured spectrophotometrically at 340 nm.

New England Nuclear Corp. supplied the [5,6-^{14}C]-DL-isocitrate. The concentration of the *threo*-D_S-isocitrate isomer was obtained by measuring the amount of TPN^+ converted to TPNH using isocitrate dehydrogenase and was found to constitute half the stated total isocitrate concentration.

Enzyme Modification. Carboxymethylmethionine enzyme was produced by incubation of 2 mg/ml of TPN–isocitrate dehydrogenase with 28 m*M* iodoacetate at pH 5.6 and 30° in 0.2 *M* sodium acetate buffer (Colman, 1968). The enzyme was dialyzed at 4° against 0.09 *M* potassium phosphate buffer (pH 6.8) and then against standard triethanolamine buffer.

Thiocyano enzyme was produced by incubation of the enzyme (1.5 mg/ml) with 2.5×10^{-4} *M* Nbs_2 for 60 min at 25° in standard triethanolamine buffer. After this time period 0.05 *M* potassium cyanide was added and the incubation was continued for 120 min. The mixture was then dialyzed against standard triethanolamine buffer at 4° until no absorption from 2-nitro-5-mercaptobenzoate could be detected in the enzyme preparation. Further reaction of treated enzyme with Nbs_2 in 0.2% sodium dodecyl sulfate revealed two sulfhydryl groups. Native enzyme when reacted with Nbs_2 in 0.2% sodium dodecyl sulfate has six reactive sulfhydryl groups; hence, four sulfhydryl groups are reacted in the thiocyano preparation. Studies (Johanson and Colman, 1974) have shown that under similar conditions of treatment, 4 mol of [^{14}C]KCN are incorporated into isocitrate dehydrogenase.

Inactive MalNEt enzyme was obtained by reaction of isocitrate dehydrogenase (2 mg/ml) with 0.3 m*M* *N*-ethylmaleimide at 30° (half-time for activity loss was 12.5 min) in standard triethanolamine buffer (Colman and Chu, 1970a). Active MalNEt enzyme was prepared by incubation with 0.3 m*M* MalNEt for 37 min under the same conditions with the addition of 2 m*M* manganous sulfate and 4 m*M* DL-isocitrate. In both cases reaction was followed by dialysis at 4° against the standard triethanolamine buffer.

Glycinamide enzyme was prepared by incubation of TPN-dependent isocitrate dehydrogenase ([MG/ml) with 0.36 *M* glycinamide and 0.064 *M* 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate in 0.03 *M* imidazole buffer (pH 7.0) for 60 min at 25° as previously described (Colman, 1973). The preparation was then dialyzed at 4° against standard triethanolamine buffer.

Ultrafiltration Binding Studies. Binding experiments with TPNH and [^{14}C]TPN^+ were conducted at 25° in 0.12 *M* triethanolamine chloride buffer (pH 7.4) containing 2% glycerol and 0.06 *M* sodium sulfate. Additional studies with TPNH and [^{14}C]isocitrate were conducted in 0.04 *M* triethanolamine buffer (pH 7.4) containing 2 or 2.5% glycerol and 0.06 or 0.075 *M* sodium sulfate. Free ligand was separated from enzyme-bound ligand by ultrafiltration through a Diaflo PM-10 membrane (Amicon Corp.) using an Amicon Model 10-PA ultrafiltration cell. In the absence of enzyme, retention of radioactivity by the membrane was less than 1%. The ^{14}C-labeled compounds were measured in Aquasol (New England Nuclear Corp.) using a Packard 3300 liquid scintillation counter. Both free and total ligand concentrations were measured. The total TPNH was determined from the absorbance at 340 nm of the initial solution. Free TPNH in the filtrate was measured by comparison of the fluorescence (see below) with dilutions of known concentrations. Bound ligand concentration in all cases was calculated from the difference between total and free concentrations.

Fluorescence Measurements. Fluorescence emission spectra were obtained on a Perkin-Elmer MPF-3 spectrofluorimeter with corrected spectra accessory. Solutions of 2.0 ml in a 1-cm^2 square cuvet were used in a thermostated cell block maintained at 25°. Fluorescence titrations with TPNH were routinely made using the uncorrected mode of the spectrofluorimeter with excitation at 340 nm and a bandwidth of 5 nm, and emission at 430 nm with a bandwidth of 16 nm. Small corrections were made for the absorbance of the TPNH solutions and for dilution by the small volumes of added TPNH. The ratio of relative emis-

[1] Abbreviations used are: MalNEt, *N*-ethylmaleimide; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

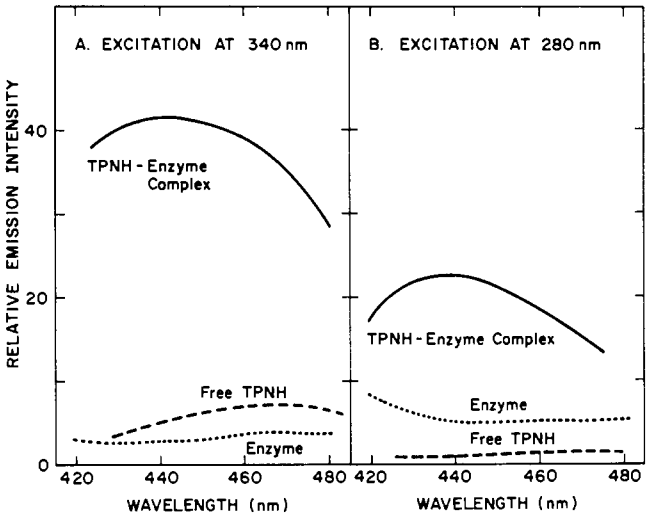

FIGURE 1: Corrected fluorescence emission spectra of TPNH and TPN-dependent isocitrate dehydrogenase resulting from (A) excitation at 340 nm and (B) excitation at 280 nm. The spectra shown are for 8.3 μM enzyme alone (. . .), 3.1 μM free TPNH (- - - -), and 3.1 μM TPNH-enzyme complex (—) dissolved in 0.04 M triethanolamine chloride buffer (pH 7.4) containing 2.5% glycerol and 0.075 M sodium sulfate. Constant slit widths of 5 nm for excitation and 8 nm for emission are maintained. The curves for the TPNH-enzyme complex have been obtained by subtracting the contribution of free TPNH (1.9 μM) and free enzyme from the spectrum of 5.0 μM TPNH plus 8.3 μM isocitrate dehydrogenase since an ultrafiltration experiment indicated that for the sample used the concentration of TPNH-enzyme complex is 3.1 μM.

sion intensity of bound TPNH to that of free TPNH was determined from the equation:

$$\text{fluorescence observed} = \begin{bmatrix}\text{fluorescence of}\\ \text{free TPNH}\end{bmatrix}\frac{(\text{TPNH})_{\text{free}}}{(\text{TPNH}_{\text{total}}} + \begin{bmatrix}\text{fluorescence of}\\ \text{bound TPNH}\end{bmatrix}\frac{(\text{TPNH})_{\text{bound}}}{(\text{TPNH})_{\text{total}}} \quad (1)$$

The fluorescence emission intensity at 430 nm was measured for samples containing 3.8–11 μM TPNH and approximately 6 μM TPN-isocitrate dehydrogenase in buffer containing 0.04 M triethanolamine chloride (pH 7.4) plus 2% glycerol and 0.06 M sodium sulfate. The fluorescence emission intensity was measured in identical samples except for the absence of enzyme. With the free and bound concentrations found from ultrafiltration experiments, eq 1 was used to calculate the fluorescence of bound TPNH. The fluorescence of bound TPNH was determined to be enhanced 10.6 ± 0.5 times relative to free TPNH. The fluorescence of bound TPNH was also determined in a series of experiments in which the fluorescence emission of 1 μM TPNH was measured as the concentration of enzyme was varied. The fluorescence was extrapolated to infinite enzyme concentration to yield the fluorescence characteristic of completely bound TPNH. An enhancement of 10.1 ± 0.7 was found by this extrapolation technique. Over the range from 0.3 to 0.6 mol of TPNH bound per mol of protein, the enhancement does not depend upon the relative saturation, thus implying the existence of a single class of binding sites. The same enhancement was found for enzyme concentrations of 4–15 μM, and for 0.04 or 0.12 M triethanolamine buffer.

Results

Fluorescence Spectra. Upon excitation at the absorption maximum, 340 nm, free TPNH exhibits a fluorescence emission spectrum with a peak at 465 nm, as shown in Figure 1A. As a result of binding of TPNH to isocitrate dehydrogenase, the emission maximum shifts approximately 25 nm to the blue and there is a marked enhancement in emission intensity, whereas the wavelength of maximum absorbance remains constant within 2 nm.

Upon excitation of the same samples at 280 nm an emission peak for isocitrate dehydrogenase alone is observed at 338 nm. The position of this peak remains invariant as the excitation wavelength is varied from 250 to 290 nm. This fluorescence spectrum is characteristic of tryptophan residues in an environment which is slightly hydrophobic (Weber and Teale, 1965). When TPNH is bound to isocitrate dehydrogenase, there is a quenching of about 60% in the enzyme fluorescence at 338 nm. At concentrations of TPNH as high as 10 μM the absorbance at 280 nm is only 0.03; thus, this effect cannot be due to an inner filter effect at the exciting wavelength. Nor can it be attributed entirely to absorbance at 340 nm by TPNH as can be seen from a similar experiment with 10 μM DPNH in which the quenching of enzyme fluorescence at 338 nm was less than 5%.

Excitation at 280 nm of solutions containing both TPNH and isocitrate dehydrogenase results in a fluorescence emission intensity at 440 nm that is greatly increased in comparison to that of free TPNH or enzyme alone (Figure 1B). This increase presumably arises from energy transfer from excited aromatic residues (mainly tryptophans) to the nicotinamide ring of TPNH (Velick, 1958).

Upon addition of 10 μM DPNH to TPN-dependent isocitrate dehydrogenase the fluorescence spectrum of DPNH (excited at 340 nm) remains unaltered from its free spectrum and there is no evidence for energy transfer from tryptophans of isocitrate dehydrogenase to DPNH.

The fluorescence emission and excitation spectra of glycinamide, carboxymethyl, and thiocyano-modified isocitrate dehydrogenases are identical with that of native enzyme. The excitation spectrum of 11% active MalNEt enzyme is altered with the appearance of a shoulder at 291 nm. The emission peak is shifted slightly from 338 nm in native enzyme to 341 nm in the 11% active MalNEt enzyme.

TPNH Binding. TPNH binding to isocitrate dehydrogenase may be followed by the enhancement of the fluorescence emission above 400 nm upon excitation at 340 or 280 nm, or by the quenching of enzyme fluorescence at 338 nm. The large enhancement of TPNH fluorescence noted in Figure 1A has been used for most measurements. For routine measurements uncorrected spectra have been used in which the peaks for free and bound TPNH are found at 450 and 430 nm, respectively, as a result of the variation in lamp intensity and photomultiplier response with wavelength. The fluorescence data were used in conjunction with a relative fluorescent enhancement of 10.6 (as described in Experimental Procedure) and eq 1 to solve for free and bound TPNH at varying total TPNH concentrations and constant enzyme concentrations.

The binding data were analyzed in terms of the Scatchard equation:

$$\frac{r}{(\text{TPNH})_{\text{free}}} = \frac{n}{K_D} - \frac{r}{K_D} \quad (2)$$

where K_D is the dissociation of the enzyme-TPNH complex; r is the number of moles of TPNH bound per mole of enzyme; and n is the number of TPNH binding sites per

Table I: Dissociation Constants Determined from TPNH Binding Experiments.

Addition	K_D Total TPNH (μM)	K_D[a] Free TPNH (μM)	K_I
None	1.45 ± 0.12	1.45 ± 0.12	
2 mM Mn^{2+}	3.5 ± 0.9	1.93 ± 0.50	
2 mM Mn^{2+} + 3.7 μM *threo*-D_S-isocitrate	14.5	8.0	1.2 μM *threo*-D_S-isocitrate
370 μM TPN^+	7.1	7.1	96 μM TPN^+

[a] Obtained by calculating the concentration of free TPNH using the computer program with the constants given in the text.

molecule of isocitrate dehydrogenase. A Scatchard plot of the binding of TPNH to isocitrate dehydrogenase is shown in Figure 2. A linear least-squares analysis yields a dissociation constant of 1.45 μM with n equal to 0.66 TPNH sites per mole of protein. A dissociation constant for TPNH from isocitrate dehydrogenase of 0.8 μM was obtained from an energy transfer experiment and a dissociation constant of 1.7 μM was obtained from an experiment based upon the quenching of protein fluorescence at 338 nm. Thus, reasonable agreement among the three methods is obtained. The agreement between fluorescence and ultrafiltration data is illustrated in Figure 2.

In the presence of manganous ion the dissociation constant for the isocitrate dehydrogenase–TPNH complex appears to increase as shown in Table I. Data obtained from both ultrafiltration and fluorescence measurements yield a dissociation constant of 3.5 μM with n equal to 0.72 mol of TPNH bound per mol of protein. The fluorescence enhancement in the presence of manganous ion is 11.2 ± 0.8, which is unchanged from that of TPNH in the absence of metal ion. TPNH is known to complex with manganous ion with an association constant of 2560 M^{-1} when the secondary phosphate is ionized and of 590 M^{-1} when that phosphate is protonated (pK = 6.1) (Colman, 1972c). The effect of manganous ion upon TPNH binding was assessed with a computer program (Cohen and Colman, 1972) which was used to calculate the concentrations of free TPNH and TPNH complexed with manganous ion. Corrections were also made for the association of manganous ion with chloride ion (Grzybowski et al., 1970) (K_A = 3.7 M^{-1}) and with sulfate ion (Atkinson and Kor, 1965) (K_A = 139 M^{-1}). If the TPNH binding data in the presence of 2 mM manganous ion are treated in terms of free TPNH a dissociation constant for the enzyme-TPNH complex of 1.93 μM is obtained (Table I). The agreement between this value and that determined in the absence of metal ion suggests that manganous ion affects TPNH binding only by means of its ability to complex TPNH, thereby decreasing the proportion of free TPNH which is available for binding to the enzyme.

As presented in Table I, when 2 mM manganous ion plus 3.7 μM *threo*-D_S-isocitrate are present, an increase is observed in the dissociation constant for the enzyme-TPNH complex which cannot be accounted for by the reduction in the concentration of free TPNH. If this is interpreted in terms of inhibition of TPNH binding by isocitrate, an inhibition constant of 1.2 μM for isocitrate is obtained which is in excellent agreement with the dissociation constant measured directly (see below and Colman, 1969a). This effect may be monitored by the decrease in the fluorescence intensity produced upon the addition of isocitrate to the enzyme in the presence of manganous ion and a constant concentration of TPNH (Figure 3). Isocitrate has little effect on the fluorescence of free TPNH but the apparent fluorescence

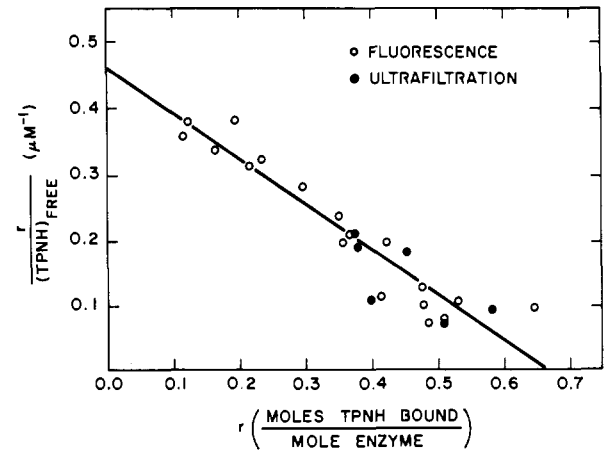

FIGURE 2: Binding of TPNH to native TPN-isocitrate dehydrogenase. Solutions contain enzyme concentrations of 6-7 μM in 0.04 M triethanolamine chloride buffer (pH 7.4) containing 2.5% glycerol, 0.075 M sodium sulfate, and 0.2 mM EDTA. TPNH concentrations range from 1 to 11 μM. Fluorescence experiments were conducted using an excitation wavelength of 340 nm and emission wavelength of 430 nm as described in Experimental Procedure. The line is a least-squares fit to the Scatchard equation with n = 0.66 ± 0.06 and K_D = 1.45 ± 0.12 μM.

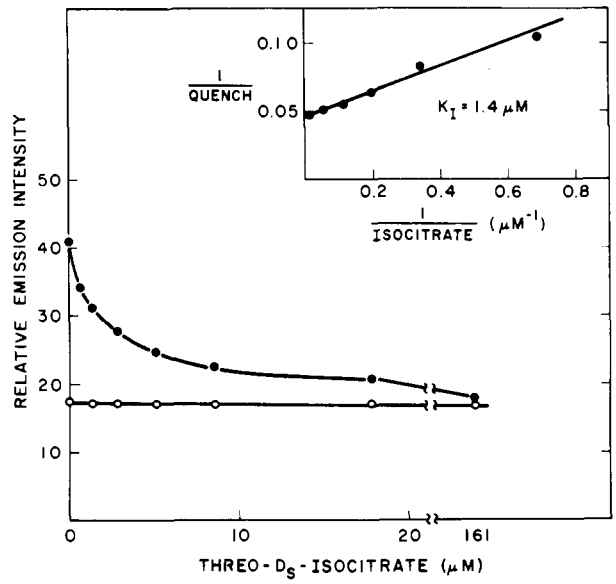

FIGURE 3: Decrease in TPNH fluorescence emission intensity by added isocitrate and manganous ion. The fluorescence emission intensity at 430 nm is measured for solutions of isocitrate dehydrogenase (1.6 μM), TPNH (4.5 μM), and manganous sulfate (2 mM) in 0.04 M triethanolamine buffer (pH 7.4) containing 2.5% glycerol, 0.075 M sodium sulfate, and the indicated total concentrations of *threo*-D_S-isocitrate. Excitation was at 340 nm. In the inset, the reciprocal of the apparent quenching (where quenching is defined as the difference between the observed emission intensity in the absence of isocitrate and observed emission intensity in the presence of isocitrate) is plotted as a function of the reciprocal of the concentration of *threo*-D_S-isocitrate. Corrections are made for changes in solutions that are identical except for the omission of isocitrate dehydrogenase. The intercept on the abscissa, obtained by extending the line, gives the total concentration of isocitrate needed to give one-half the maximum reduction in emission intensity. The intercept on the ordinate is equal to the reciprocal of the difference in fluorescence emission intensity of enzyme and buffer solutions.

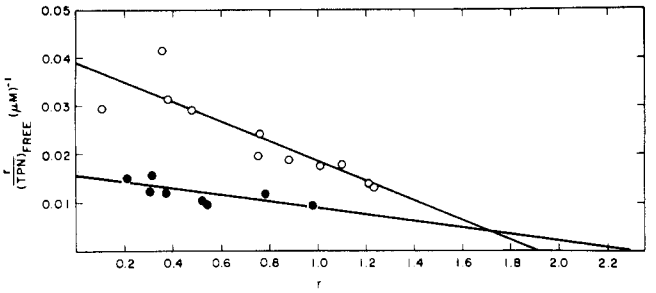

FIGURE 4: Binding of carbonyl [^{14}C]TPN$^+$ by native isocitrate dehydrogenase in the presence (●) and absence (○) of TPNH. The solutions contain 4–14 μM isocitrate dehydrogenase in 0.12 M triethanolamine chloride (pH 7.4) containing 0.06 M sodium sulfate and 2% glycerol. In the absence of TPNH the data are fit to the Scatchard equation with n = 1.92 ± 0.17 and K_D = 49 ± 9 μM. In the presence of TPNH a fit to the Scatchard equation with n = 2.29 ± 0.27 and K_D = 149 ± 14 μM is shown.

Table II: TPNH Binding by Native and Chemically Modified Isocitrate Dehydrogenase.

Enzyme Preparation	Residual Activity[a] (%)	K_D (μM)	n (moles of TPNH Bound / mole of enzyme)
Native	100	1.45	0.66 ± 0.06
Glycinamide	11		0.09
Thiocyano	11	1.5	0.07 ± 0.01
Carboxymethyl	11	1.8	0.06 ± 0.01
MalNEt	11	0.6	0.25 ± 0.03
	24	0.7	0.35 ± 0.09
MalNEt (protected)[b]	100	0.8	0.5 ± 0.1

[a] Activity remaining relative to initial activity. [b] Protected with 4 mM DL-isocitrate and 2 mM Mn^{2+}.

intensity of the bound TPNH is decreased to the level of free coenzyme. That this decreased fluorescence is due to reduced TPNH binding and not to a quenching of the characteristic fluorescence of bound TPNH was confirmed by an ultrafiltration experiment which indicated that no TPNH was bound in the presence of saturating concentrations of isocitrate and manganous ion. The inset in Figure 3 shows a double reciprocal plot of the effect of isocitrate on TPNH binding. These data extrapolate to no bound TPNH at infinite concentration of isocitrate and yield an inhibition constant of 1.4 μM for *threo*-Ds-isocitrate in the presence of manganous ion. Isocitrate in the absence of manganous ion causes a decrease in the fluorescence of the enzyme-bound TPNH with an apparent inhibition constant of 4000 μM. The data do not extrapolate to completely free TPNH at infinite concentration of isocitrate and follow closely the response to citrate which is a weak inhibitor of isocitrate dehydrogenase.

In the presence of 370 μM TPN$^+$ the dissociation constant for TPNH is increased to 7.1 μM (Table I), which allows calculation of an apparent inhibition constant for TPN$^+$ of 96 μM. An inhibition constant for TPN$^+$ of 77 μM was obtained from a measurement of the TPNH fluorescence at a constant TPNH concentration with increasing concentrations of TPN$^+$. At infinite concentration of TPN$^+$ the amount of TPNH bound extrapolates to less than 0.05 mol/mol of protein. A similar titration with DPN$^+$ yields an inhibition constant two orders of magnitude greater, 13000 μM, which is consistent with the specificity of the enzyme for TPN$^+$.

Binding of TPNH to Chemically Modified Isocitrate Dehydrogenases. The ability of native and chemically modified isocitrate dehydrogenases to bind TPNH has been tested by the fluorescence titration and ultrafiltration techniques. The results are recorded in Table II. The following modified enzymes retain approximately 11% of their original catalytic activity: glycinamide enzyme with one altered glutamyl residue, thiocyano enzyme with an average of four modified cysteinyl residues, and carboxymethyl enzyme with one altered methionyl residue. In each case, the number of TPNH binding sites is less than 0.09 on the basis of Scatchard plots of the fluorescence titrations; these results have been confirmed by ultrafiltration experiments. This number is consistent with binding solely to the approximately 11% active enzyme. The dissociation constants measured are in reasonable agreement with that of the native enzyme, suggesting that the measured TPNH binding represents that from the residual unmodified native enzyme.

N-Ethylmaleimide inactivates isocitrate dehydrogenase by reaction with an average of two cysteinyl residues although it has been postulated that only one of these is essential for activity (Colman and Chu, 1970b). Loss of the ability to bind TPNH does not follow precisely the extent of inactivation in this case (Table II). At 11 or 24% residual activity, the amount of TPNH bound is somewhat greater than that to be expected for this residual activity. Partially active preparations must represent heterogeneous mixtures in which none, either one, or both of the two sulfhydryl groups have reacted with MalNEt; and it seems likely that reaction only with the cysteinyl residual essential for activity does not totally eliminate the ability to bind TPNH.

It has been reported (Colman and Chu, 1970b) that when isocitrate dehydrogenase is incubated with MalNEt in the presence of isocitrate and manganous ion an enzyme is produced which retains 100% of its original activity as measured under standard assay conditions but which has an average of two modified cysteinyl residues. The two residues altered under these conditions are not the same as those modified in the absence of substrates. As shown in Table II, the binding of TPNH by this 100% active MalNEt enzyme (protected) is only slightly different from that of the native enzyme. Since the measurements made with this enzyme are subject to greater than normal systematic errors due to the lability of this preparation, it is not certain that this difference is significant. The enhancement of TPNH fluorescence in this enzyme is the same as that for native enzyme. The 100% active MalNEt modified enzyme was also titrated with isocitrate in the presence of 2 mM manganous ion and constant TPNH and the decrease in TPNH fluorescence was followed. The data were treated in the same manner as shown for native enzyme in Figure 4 and an inhibition constant for *threo*-Ds-isocitrate of 10 ± 2.5 μM was found as compared with 1.4 μM for native unmodified enzyme. Kinetic studies (Colman and Chu, 1970a) have also shown an increase in the Michaelis constant for isocitrate with both 100% active MalNEt modified enzyme and with partially active preparations.

TPN$^+$ Binding to Native Isocitrate Dehydrogenase. The binding of carbonyl [^{14}C]TPN$^+$ to native isocitrate dehydrogenase was measured using the ultrafiltration technique, and a Scatchard plot of these binding data is shown in Figure 4. The data can be fit by a single line for a dissociation

Table III: Binding of [^{14}C]TPN$^+$ by Isocitrate Dehydrogenase.

Enzyme Preparation	K_D (μM)	n
Native enzyme	49 ± 9	1.92 ± 0.17
Carboxymethyl enzyme	120 ± 50	2.3 ± 0.5
Thiocyano enzyme	150 ± 100	2.2 ± 1.0
Glycinamide enzyme	440 ± 330	3.0 ± 1.5

constant of 49 μM and 1.92 TPN$^+$ binding sites per molecule of enzyme. This constant is 10 times higher than the reported Michaelis constant for TPN$^+$ (Colman, 1968). Thus, attempts were made to ascertain whether the binding of TPN$^+$ is strengthened upon the addition of isocitrate alone, manganous ion plus citrate, or manganous ion plus α-ketoglutrate. The experiments with isocitrate were conducted in the presence of 15 mM EDTA in order to prevent the reduction of TPN$^+$ by enzyme activated by residual metal in the buffer. None of the additions tested enhanced or altered appreciably the binding of TPN$^+$.

A reduction in the binding of TPN$^+$ is produced by 15 μM TPNH, as shown in Figure 4. Although the free TPNH concentration in these experiments is not constant but varies from 11 to 13 μM, the data can as a good approximation be fit by a straight line giving a dissociation constant for the enzyme–TPN$^+$ complex of 149 μM with 2.29 binding sites. The binding of TPNH appears to reduce the binding of TPN$^+$ on both sites. On the basis of the assumption that the bindings of TPN$^+$ and TPNH to the enzyme are mutually exclusive an inhibition constant for TPNH of approximately 4 μM is calculated.

TPN$^+$ Binding to Modified Isocitrate Dehydrogenase. Results of TPN$^+$ binding experiments with carboxymethyl-, thiocyano-, and glycinamide-modified isocitrate dehydrogenase are summarized in Table III. In the cases of carboxymethyl and cyano enzyme the dissociation constant is increased only two- to threefold, while for the glycinamide-modified enzyme the increase is close to tenfold. For all these modified enzymes the number of binding sites appears unchanged. The accuracy of these measurements is limited by the weakness of the binding and the possibility that the two sites observed in the native enzyme are unequally affected by modification cannot be excluded.

Isocitrate Binding to Native Isocitrate Dehydrogenase. The binding of [5,6-^{14}C]-DL-isocitrate to native enzyme was studied to determine whether the effect of isocitrate on TPNH binding was reciprocal. In the presence of 2 mM manganous ion, 0.98 mol of *threo*-Ds-isocitrate is bound with a dissociation constant of 1.25 μM as shown in the Scatchard plot of Figure 5. This is in agreement with previous results (Colman, 1969a) and the inhibition constant found from the study of TPNH binding above. In the absence of manganous ion the number of isocitrate binding sites remains unchanged, but the dissociation constant for isocitrate is increased to 34 μM. This dissociation constant is about six times that measured previously (Colman, 1969a); the reason for this difference is not known. The reduction of isocitrate binding by two concentrations of TPNH for two isocitrate concentrations is shown in Figure 5. The effect of TPNH in weakening the binding of manganese–isocitrate indeed reciprocates the weakening of TPNH binding by manganese–isocitrate. No effect of TPNH on isocitrate binding in the absence of manganous ion was detected. From a number of measurements in which isocitrate

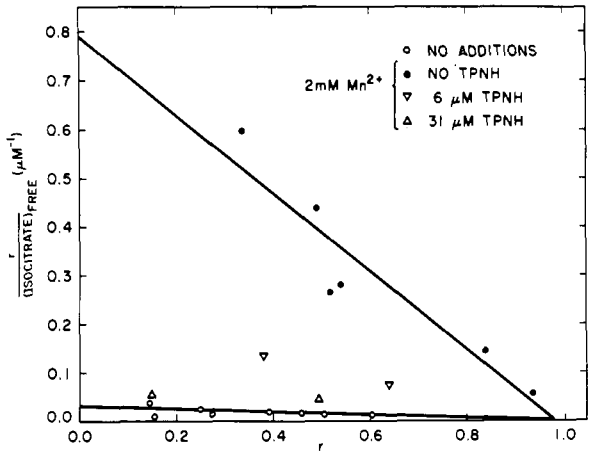

FIGURE 5: Binding of [5,6-^{14}C]isocitrate to native isocitrate dehydrogenase in the presence (△,▽) and absence (●,○) of TPNH. The binding was measured by the ultrafiltration technique using enzyme concentrations from 2.8 to 12.5 μM in 0.04 M triethanolamine chloride buffer (pH 7.4) containing 0.06 M sodium sulfate, 2% glycerol, and concentrations of manganous sulfate and TPNH as indicated. The line through the points in the absence of manganous ion and TPNH is a least-squares fit to the Scatchard equation with n = 1.0 ± 0.1 and K_D = 34 ± 14 μM. A fit to the data in the presence of manganous ion but in the absence of TPNH yields n = 0.98 ± 0.08 and K_D = 1.25 ± 0.24 μM.

binding in the presence of manganous ion and TPNH was measured, an inhibition constant for TPNH of 2.7 ± 0.6 μM was calculated. This compares favorably with the dissociation constant of 3.5 μM measured for the enzyme–TPNH complex in the presence of manganous ion (Table I).

Binding of [^{14}C]Isocitrate to Chemically Modified Isocitrate Dehydrogenase. The binding of isocitrate to the thiocyano- and glycinamide-modified isocitrate dehydrogenases was measured in comparison to the native enzyme. For both modified enzymes, approximately 1 mol of isocitrate is bound per peptide chain. The binding is equally weak regardless of whether manganous ion is added; the dissociation constant for these inactive enzymes, in the presence or absence of manganese, is essentially the same as that observed for the native enzyme in the absence of manganese (34 μM). The binding of isocitrate to the carboxymethyl enzyme has been studied previously (Colman, 1969a), and in contrast to the modifications studied here, the enzyme with an altered methionyl residue does show enhanced binding of isocitrate in the presence of metal ion.

Discussion

A marked change in the fluorescence of TPNH occurs upon binding to TPN-dependent isocitrate dehydrogenase, the emission maximum shifting 20 nm to the blue and the emission intensity increasing about tenfold. A similar shift in the fluorescence spectrum accompanied by an enhancement in the emission intensity has been noted for DPNH binding to DPN-dependent isocitrate dehydrogenase (Harvey et al., 1972) as well as other dehydrogenases (Winer and Theorell, 1960; Perkins and Bertino, 1966; Hsu and Lardy, 1967). Energy transfer from aromatic residues to bound TPNH is noted with accompanying quenching of the protein fluorescence of isocitrate dehydrogenase. Since the characteristic distance for energy transfer from tryptophans to pyridine nucleotides is of the order of 25 Å (Karreman et al., 1958), this interaction is not surprising and does not

necessarily indicate the presence of an aromatic residue at the coenzyme binding site. There is no shift in the protein fluorescence maximum; thus, different tryptophans cannot be distinguished as in the case of the DPN-linked isocitrate dehydrogenase from bovine heart (Fan et al., 1975).

The altered fluorescence properties of the enzyme–coenzyme complex have permitted exploration of the interaction of the coenzyme with isocitrate dehydrogenase in the presence or absence of other ligands. Studies of TPNH binding to native enzyme in the presence and absence of manganous ion suggest that it is free TPNH, rather than the metal complex, which binds to the enzyme. The measured dissociation constant of 1.45 μM for free TPNH is less than the Michaelis constant of 12 μM (Colman, 1973). The higher value for the Michaelis constant results in part from the complexing of TPNH by the 2 mM manganous ion normally present in the assay solution. Kinetic studies (Uhr et al., 1974) have indicated that there is competition between bicarbonate or carbon dioxide and TPNH and this competition would also cause the observed Michaelis constant to be higher than the actual dissociation constant. When the effects of competing ligands are taken into account, there is reasonably close agreement between the measured dissociation constant and Michaelis constant and it is probable that the binding site observed is that involved in catalysis.

A single TPNH site has been observed in the present investigation which utilizes a homogeneous enzyme preparation. Even at TPNH concentrations as high as 100 μM no more than 1 mol of TPNH is bound. This result contrasts with earlier estimates of two sites per molecule (Langan, 1960). In the previous study the isocitrate dehydrogenase was only partially purified, having a specific activity of about 5 enzyme units per milligram, as compared with 30 for the pure protein.

In the present investigation it has been shown that TPNH binding to native isocitrate dehydrogenase in the presence of manganous ion plus isocitrate and isocitrate binding in the presence of manganous ion plus TPNH are in quantitative agreement. The reduced coenzyme and substrate sites are mutually exclusive. Supportive evidence for this conclusion comes from the detritiation reaction of α-ketoglutarate which was studied by Rose (1960). In these experiments it was shown that TPNH was necessary for the ditritiation of α-ketoglutarate in the presence of magnesium ion. The rate of detritiation was reduced substantially by the addition of isocitrate but the inhibition could be compensated for by an increase in the TPNH concentration. The present finding that the binding of TPNH and metal–isocitrate is mutually exclusive readily explains these results. Rose (1960) has postulated that TPNH maintains the enzyme in a conformation suitable for binding of α-ketoglutarate at the active site. It is possible that manganese–isocitrate is unable to bind to this configuration of the enzyme and hence the interaction between TPNH and substrate is indirect without overlap of the binding sites. Nevertheless, it is quite probable that the binding sites for substrate and reduced coenzyme overlap. The nicotinamide ring must be close to the substrate site in order to participate in hydride transfer from substrate to oxidized coenzyme. Upon reduction, the orientation of the nicotinamide ring could change so as to overlap the substrate site.

The binding experiments with carbonyl [^{14}C]TPN$^+$ show two binding sites per 58000 molecular weight peptide chain. These data contrast with the measurements of a single binding site for TPNH, isocitrate, α-ketoglutarate (Colman, 1969a), and manganese (Villafranca and Colman, 1972), which imply that there is only one active site in the enzyme. The two sites for oxidized coenzyme appear to bind TPN$^+$ with the same dissociation constant of 49 μM which is ten times higher than the Michaelis constant of 4.6 μM (Colman, 1968). Langan (1960) had also observed that TPN$^+$ binds weakly compared with TPNH. The question arises as to the relationship of the binding sites observed here to the active site. Kinetic studies have been interpreted to imply a random mechanism for isocitrate dehydrogenase (Uhr et al., 1974). Such a mechanism requires that TPN$^+$ bind independently of substrate and thus the dissociation constant for TPN$^+$ should be equal to its Michaelis constant or smaller (since the kinetic studies require the presence of divalent metal which chelates TPN$^+$ and reduces its free concentration). These experiments on the binding of TPN$^+$ exclude a random mechanism for isocitrate dehydrogenase as well as an ordered mechanism in which TPN$^+$ binds first. The weak binding in the absence of manganese–isocitrate implies that substrate must influence the binding of TPN$^+$ although this could not be tested directly. The failure of α-ketoglutarate to alter the dissociation constant of the enzyme–TPN$^+$ complex is consistent with the kinetic evidence (Uhr et al., 1974; Northrop and Cleland, 1974) which shows that inhibition by α-ketoglutarate is not due to formation of a dead-end enzyme–TPN$^+$–α-ketoglutarate complex.

The relationship between the TPN$^+$ and TPNH binding sites of native isocitrate dehydrogenase is elucidated by several different types of experiments. The addition of TPNH appears to disrupt the binding of TPN$^+$ at both sites and raises the possibility that the action of TPNH is via a change in enzyme conformation which renders TPN$^+$ binding less favorable. Under assay conditions, the presence of TPNH up to 40 μM (TPN$^+$ concentration is then 60 μM) causes little change in the reaction velocity. This supports the suggestion that the binding of TPN$^+$ observed in the direct binding experiments is not the same as the binding that occurs in the presence of substrates. The decrease in the binding of TPN$^+$ in the presence of TPNH allows the calculation of an inhibition constant for TPNH of approximately 4 μM, which is almost three times the dissociation constant measured directly. This result might be explained if TPNH does not block the binding of TPN$^+$ but merely changes the affinity for TPN$^+$ of either one or both sites to a much lower level. Unfortunately, it is not possible to distinguish between these possibilities because of the difficulty in measuring small differences in the weak TPN$^+$ binding. The oxidized coenzyme has been found to inhibit the binding of TPNH. There is no evidence for a conformational change in isocitrate dehydrogenase upon binding TPN$^+$ but TPN$^+$ could prevent a conformational change accompanying TPNH binding. It is also possible that one or both of the TPN$^+$ binding sites overlaps the TPNH site. The strong specificity for the 2′-phosphoadenosine moiety makes it likely that overlap occurs at that portion of the coenzyme binding site. Besides the binding evidence presented here, there is additional evidence for a difference in either the configuration of the bound reduced and oxidized coenzymes or the configuration of the respective enzyme–coenzyme complexes. For example, protection against inactivation by diazo-1H-tetrazole is afforded by TPNH but not TPN$^+$ (Colman, 1972a). Protection against modification by Nbs_2 is afforded by TPNH alone and will be discussed below. The hypothesis that the configuration of the reduced coen-

zyme-enzyme complex is altered is supported by the stimulation of detritiation of α-ketoglutarate which is not observed for the TPN^+-enzyme complex (Rose, 1960). Direct evidence for a configurational change has not been found from the fluorescence of enzyme aromatic groups studied here.

In general, modification of amino acid residues of TPN-isocitrate dehydrogenase with resultant loss of enzyme activity is characterized by a loss of TPNH binding. Protein fluorescence provides no evidence for a change in conformation upon enzyme modification. As in the case of detritiation of α-ketoglutarate, conformational changes cannot be ruled out. Modification of a single glutamyl residue in the glycinamide enzyme results in a loss of both TPNH and manganese-isocitrate binding capacity with manganese binding in the absence of isocitrate (Colman, 1973) and isocitrate binding in the absence of manganous ion remaining unimpaired. These binding data may be compared with the effect of the same ligands on the rate constant for formation of the inactive glycinamide enzyme: a small decrease in the rate constant is provided by incubation with TPNH, while apparently complete protection is provided by manganese-isocitrate. Small reductions in the rate of inactivation are provided by TPN^+, manganous ion, or isocitrate alone. Lack of protection by coenzyme implies that the loss of TPNH binding and the tenfold increase in the dissociation constant for TPN^+ are indirect effects and modification is not at the coenzyme binding site. In contrast, the specific protection against modification of the glutamyl residue by manganese-isocitrate, coupled with the loss of manganese-isocitrate binding, suggests that the primary function of the modified glutamyl residue may be to participate in the binding of substrate.

The thiocyano enzyme, prepared by successive reaction of isocitrate dehydrogenase with Nbs_2 and cyanide, shows a loss of the ability to bind TPNH and managanese-isocitrate; whereas, binding of isocitrate alone or manganese alone (Johanson and Colman, 1974) is not impaired. The rate of inactivation by Nbs_2 can be substantially decreased by manganous ion plus TPNH but not TPNH or isocitrate alone (Colman, 1969b). The addition of TPN^+, either alone or with manganous ion, has no effect on the rate of inactivation. In view of the evidence that manganous ion is not required for TPNH binding and, indeed, competes with isocitrate dehydrogenase for free TPNH, it is surprising that manganous ion is required for protection by TPNH. The protection is not specific for any of the modified sulfhydryl groups, but instead decreases the rate of reaction of Nbs_2 at all cysteine residi s (Johanson and Colman, unpublished data). It may be that this nonspecific effect results from a conformational change in the enzyme produced by TPNH, combined with protection by manganous ion in the new configuration. The decreased number of reacted sulfhydryl groups after 120 min of incubation with Nbs_2 plus TPNH compared with the number reacted in the presence of Nbs_2 alone is evidence that TPNH reduces the reactivity of sulfhydryls not at the active site (since the rate of loss of activity is the same with or without TPNH) (Colman, 1969b). Thus, as for the glycinamide enzyme, the thiocyano enzyme shows no evidence that the modification responsible for the loss of activity is within the coenzyme binding site. The significance of the relatively small increase in the TPN^+ dissociation constant for the thiocyano enzyme is questionable since the sites observed in the TPN binding experiments are not necessarily related to the active site. In contrast to coenzyme, manganese-isocitrate produces a striking decrease in the rate of inactivation of the enzyme by Nbs_2 as well as a decrease in the modification of specific cysteinyl residues (Johanson and Colman, 1974). It may be that the primary role of the critical cysteinyl residues is also to participate in the specific tight binding of manganese-isocitrate. The loss of TPNH binding may be a secondary effect which is again a manifestation of the interrelationship between the binding of substrate and reduced coenzyme.

In the MalNEt-modified enzyme loss of activity is not directly related to loss of TPNH binding. This may be attributed to the heterogeneity of the preparations in which an average of two cysteinyl groups are modified at somewhat different rates. Modification of one of the groups which is necessary for activity does not totally preclude TPNH binding. The MalNEt enzyme shows alterations in the protein fluorescence emission and excitation spectra. Previously (Colman and Chu, 1970b) a change in the optical rotatory dispersion was found. These spectral changes indicate an altered conformation in the modified enzyme even for the 100% active (manganese-isocitrate protected) enzyme. This altered conformation is also reflected in the change in the binding constant for isocitrate in the presence of manganese for the 100% active MalNEt enzyme determined here from its competitive effect on TPNH binding (and in agreement with the altered Michaelis constant reported previously (Colman and Chu, 1970a)).

The inactivation by MalNEt is subject to substantial protection only by the substrate, manganese-isocitrate. Evidence that the TPNH binding site may lie near but not at the active site sulfhydryl comes from protection by TPNH obtained against modification by the bulky *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide derivative but not by the smaller *N*-ethylmaleimide. This protection by TPNH could be indirect as is probably the case for protection against Nbs_2 inactivation. The inactive MalNEt enzyme has been shown to be incapable of binding manganese-isocitrate (Villafranca and Colman, 1972), and, again, this defect may be the primary reason for the inactivation of this cysteinyl-inactivated enzyme.

Carboxymethylation of a single methionine of isocitrate dehydrogenase results in loss of TPNH binding. In this altered enzyme, binding by substrate is retained (Colman, 1969a; Villafranca and Colman, 1972). Approximately a tenfold reduction in the rate of inactivation by iodoacetate is obtained with saturating concentrations of isocitrate in the presence or absence of manganous ion. Failure to obtain a greater decrease in the rate of inactivation and retention of the ability of the modified enzyme to bind substrate indicate that reaction with iodoacetate does not take place directly at the substrate site. Only a slight increase in the TPN^+ dissociation constant is observed in the modified enzyme. Assuming that the TPN^+ binding site is not the same as that which obtains in the active complex it is possible that the loss in activity upon reaction of a methionyl residue with iodoacetate arises from disruption of the coenzyme binding site; however, lack of protection by TPNH would make this improbable and the hypothesis that the methionyl residue is involved in catalysis (Colman, 1968) remains as more likely.

In examining several modifications of isocitrate dehydrogenase it is seen that the binding of TPNH and manganese-isocitrate are closely related. The carboxymethyl enzyme is an exception and it may be that TPNH binding is lost because the modified enzyme is unable to assume the altered

configuration postulated to arise upon binding TPNH (Rose, 1960). Alternatively, it may be postulated that the manganese–isocitrate and TPNH sites overlap and the region of overlap includes the glutamyl residue and critical cysteinyl residues but not the methionine residue. It is more likely that none of the modifications studied are at residues within the coenzyme binding site since in no case is specific protection provided by either oxidized or reduced coenzyme. The nature of the TPN^+ binding site is difficult to evaluate since the binding of TPN^+ during the catalytic process appears to depend upon prior binding of substrate. Hence, any of the modifications that result in loss of substrate binding would preclude tight binding of TPN^+. One possibility that might be considered is that TPN^+ also requires a change in enzyme conformation which can only occur when the substrate is bound or alternatively the conformation of bound TPN^+ must be altered to a conformation at the nicotinamide ring similar to that of TPNH. Such a change in the conformation of oxidized coenzyme, involving a nucleophile (which could be provided by enzyme or substrate), has been suggested by Velick (1958). With this hypothesis, the inactivation seen in the carboxymethyl enzyme might arise from prevention of a conformational change needed to form the active complex and the modified methionine need not be at the active site itself. Distinction among the various possibilities awaits further studies of the relationship between modified residues and the active site.

References

Atkinson, G., and Kor, S. K. (1965), *J. Phys. Chem. 69,* 128.

Cohen, P. F., and Colman, R. F. (1972), *Biochemistry 11,* 1501.

Colman, R. F. (1968), *J. Biol. Chem. 243,* 2454.

Colman, R. F. (1969a), *Biochim. Biophys. Acta 191,* 469.

Colman, R. F. (1969b), *Biochemistry 8,* 888.

Colman, R. F. (1972a), *J. Biol. Chem. 247,* 215.

Colman, R. F. (1972b), *J. Biol. Chem. 247,* 6727.

Colman, R. F. (1972c), *Anal. Biochem. 46,* 358.

Colman, R. F. (1973), *J. Biol. Chem. 248,* 8137.

Colman, R. F., and Chu, R. (1970a), *J. Biol. Chem. 245,* 601.

Colman, R. F., and Chu, R. (1970b), *J. Biol. Chem. 245,* 608.

Fan, C. C., Tomcho, L. A., and Plaut, G. W. E. (1975), *Biochem. Biophys. Res. Commun. 62,* 933.

Grzybowski, A. K., Tate, S. S., and Datta, S. P. (1970), *J. Chem. Soc. A,* 241.

Harvey, R. A., Heron, J. I., and Plaut, G. W. E. (1972), *J. Biol. Chem. 247,* 1801.

Hsu, R. Y., and Lardy, H. A. (1967), *J. Biol. Chem. 242,* 527.

Johanson, R. A., and Colman, R. F. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1444.

Karreman, G., Steele, R. H., and Szent-György, A. (1958), *Proc. Natl. Acad. Sci. U.S.A. 44,* 140.

Langan, T. A. (1960), *Acta Chem. Scand. 14,* 936.

Northrop, D. B., and Cleland, W. W. (1974), *J. Biol. Chem. 249,* 2928.

Perkins, J. P., and Bertino, J. R. (1966), *Biochemistry 5,* 1005.

Rose, Z. B. (1960), *J. Biol. Chem. 235,* 928.

Uhr, M. L., Thompson, V. W., and Cleland, W. W. (1974), *J. Biol. Chem. 249,* 2920.

Velick, S. F. (1958), *J. Biol. Chem. 223,* 1455.

Villafranca, J. J., and Colman, R. F. (1972), *J. Biol. Chem. 247,* 209.

Weber, G., and Teale, F. W. J. (1965), in The Proteins, Vol. III, Neurath, H., Ed., New York, N.Y. Academic Press, p 445.

Winer, A. D. (1964), *J. Biol. Chem. 239,* 3598.

Winer, A. D., and Theorell, H. (1960), *Acta Chem. Scand. 14,* 1729.