Reduced Pyridine Nucleotide Binding to Beef Liver and Dogfish Liver Glutamate Dehydrogenases†

Dean A. Malencik‡ and Sonia R. Anderson*

ABSTRACT: The binding of reduced pyridine nucleotides to beef liver glutamate dehydrogenase and dogfish glutamate dehydrogenase has been examined by fluorescence and circular dichroism. Beef liver glutamate dehydrogenase binds 6 moles of NADPH both in the presence and absence of GTP. This is consistent with the hexamer hypothesis for the oligomer. In agreement with previous evidence, there are multiple sites for NADH. In the presence of GTP, 18 sites/hexamer have been fluorometrically titrated and confirmed by sedimentation measurements. Formation of the abortive ternary complex indicates that six of these sites are equivalent to the NADPH binding sites. The coenzyme binding equilibrium of beef liver glutamate dehydrogenase is independent of protein concentration in the range examined (0.2–2 mg/ml). Dogfish glutamate dehydrogenase, a nonassociating form from a primitive vertebrate, is NAD^+ (H) specific but shows the same heterogeneity in the binding of NADH.

L-Glutamate dehydrogenase (EC 1.4.1.3) from beef (BL-GDH)[1] is one of the better known pyridine nucleotide dependent dehydrogenases. The amino acid sequence of the monomer (Smith *et al.*, 1970) and accurate determinations of the molecular weight of the oligomer (Eisenberg and Tomkins, 1968; Cassman and Schachman, 1971) have been recently reported.

There are numerous reports on the stoichiometry of coenzyme binding, both for NADH and NADPH (Frieden, 1964). The values for NADH range from 2 to 10 moles bound per 320,000 g[2] of protein. Some of the reports indicate that the absolute number of coenzyme molecules bound varies with the degree of protein association (Sund, 1968) and that the fluorometrically determined dissociation constants for the binary complex differ tenfold from those determined kinetically (Frieden, 1963a).

However, there are two major difficulties in the interpretation of these binding studies. The first is the report that BL-GDH binds more than one molecule of NADH per subunit (Frieden, 1970). The second is the common failure to reach stoichiometric addition in fluorescence titrations.

In this paper we will describe fluorescence and circular dichroism studies of NADH and NADPH binding to BL-GDH, both in the presence and absence of the effectors GTP and ADP. The unambiguous determination of stoichiometry and dissociation constants for the reduced coenzymes may elucidate the function of any multiple sites and the complex interactions of the enzyme with effectors.

We will examine the role of association in coenzyme binding through parallel experiments with Pacific dogfish GDH (DF-GDH), a nonassociating form of the enzyme (Corman *et al.*, 1967). Comparative studies of these two enzymes are interesting since the dogfish is a primitive vertebrate, having diverged from the main vertebrate evolutionary line about 400 million years ago.

Materials

BL-GDH was generally obtained as an ammonium sulfate suspension from Sigma Chemical Co. An ammonium sulfate suspension from Boehringer and a phosphate–glycerol solution from Sigma Chemical Co. were also used and found to have the same properties.

DF-GDH, from the Pacific dogfish (*Squalus acanthias*), was prepared by the method of Corman *et al.* (1967) with modifications. A heat treatment, 58° for 5 min, in 5% (w/v) sodium sulfate was used in the first step. All other procedures were carried out as previously reported.

Before experiments, the enzymes were dialyzed against 0.05 M potassium phosphate (pH 7.6), containing 10^{-4} M EDTA. In order to remove tightly bound nucleotides, the dialyzed enzymes were treated with 1 mg/ml of Norit A for 30 min, and then filtered through a 0.45 μ Millipore filter (Cross and Fisher, 1970).

The concentration of BL-GDH was determined using the extinction coefficient 0.97 cm^2/mg at 279 nm (Frieden, 1963a). The DF-GDH showed some absorbance above 300 nm due to a contaminant which could not be removed by either Norit treatment or recrystallization. We calculated an extinction coefficient of 1.10 ± 0.04 cm^2/mg at 280 nm for DF-GDH using the protein concentrations obtained from both total nitrogen determinations and synthetic boundary procedures using BL-GDH as a standard (Babul and Stellwagen, 1969).

NADH, NADPH, NAD^+, $NADP^+$, ADP, GTP, coenzyme analogs, and substrates were purchased from either Sigma Chemical Co. or P-L Biochemicals.

All of the experiments were conducted in 0.05 M potassium phosphate buffer (pH 7.6), containing 10^{-4} M EDTA and prepared from glass distilled water. The temperature was 20°.

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received February 15, 1972.* This work was supported by grants from the National Institutes of Health (AM 13912) and the Oregon Heart Association.

‡ U. S. Public Health Service Predoctoral trainee (GM 00253).

* Career Development awardee (GM 24134) of the National Institutes of Health.

[1] Abbreviations used are: BL-GDH, glutamate dehydrogenase from beef liver; DF-GDH, glutamate dehydrogenase from dogfish liver; $\bar{n}$, average number of moles of ligand bound per mole of protein; F_∞/F_0, the ratio of the fluorescence intensities of completely bound and free ligand measured at equal concentrations.

[2] One mole of protein is defined as 320,000 g (Cassman and Schachman, 1971).

TABLE I: Determinations of F_∞/F_0.[a]

	Without GTP	With 300 μM GTP
BL-GDH + NADH	4.3 ± 0.1	5.7 ± 0.1
BL-GDH + NADPH	5.5 ± 0.1	5.6 ± 0.1
DF-GDH + NADH	2.6 ± 0.3	4.4 ± 0.2
FD-GDH + NADPH	1.0	1.0

[a] Excitation: 340 nm; emission, 430 nm. Conditions: 0.05 M potassium phosphate–10^{-4} M EDTA, pH 7.6 (20°).

Methods

Assay. All rate measurements were made on a Cary 15 spectrophotometer using the 0–0.1 scale. The assay of Strecker (1955), based on the reduction of NAD^+ by glutamate, was used. The glutamate dehydrogenases used in these studies had specific activities of 3.5 ± 0.5 units/mg.

Fluorescence. All of the fluorescence measurements were made using the Hitachi-Perkin-Elmer MPF-2A fluorescence spectrophotometer. The procedures for the fluorescence titrations were described by Anderson and Weber (1965). The wavelengths of excitation and emission were 340 and 430 nm, respectively.

F_∞/F_0, the ratio of the fluorescence intensities of the completely bound and the free coenzyme, was determined in the following way. A solution containing 5 μM reduced pyridine nucleotide and 5 mg/ml of enzyme was initially prepared. This solution was then diluted with buffer containing 5 μM coenzyme so that a series of protein concentrations ranging from 0.5 to 5 mg per ml was obtained. The fluorescence was measured at each dilution. The observed intensities were corrected for the protein and buffer blank fluorescence. In the presence of 300 μM GTP, the fluorescence reached a constant value at protein concentrations greater than 2 mg/ml. This plateau indicated that all of the coenzyme was bound and that the maxiumum fluorescence corresponded to F_∞. In the absence of GTP, there was no plateau; F_∞ was obtained by extrapolation of a plot of $1/F$ *vs.* $1/[P]$ to $1/[P] = 0$.

Since high concentrations (>2 mg/ml) of DF-GDH have significant absorbance at 340 nm, the intensities were corrected for absorption of incident light by the enzyme using the relation

$$F_{cor} = F_{obsd} \frac{[1 - 10^{-OD_1}]}{[1 - 10^{-(OD_1 + OD_2)}]} \frac{[OD_1 + OD_2]}{OD_1}$$

where the subscripts 1 and 2 refer to the optical densities of the coenzyme and the protein at 340 nm. This factor ranged between 1.98 and 1.16 for DF-GDH. Any correction for BL-GDH was less than 1.1 and was ignored.

Sedimentation. Sedimentation experiments were carried out using the Spinco Model E ultracentrifuge equipped with scanner. The rotor speed and temperature were typically 48,000 rpm and 20°, respectively.

Circular Dichroism. All circular dichroism spectra were recorded between 300 and 400 nm on the Jasco Model CD-SP circular dichroism recorder and spectrophotometer. The absorbancy of all samples was kept below 1. Cuvets with light paths ranging from 1 mm to 10 cm were used. Base lines were determined using solutions of GDH alone.

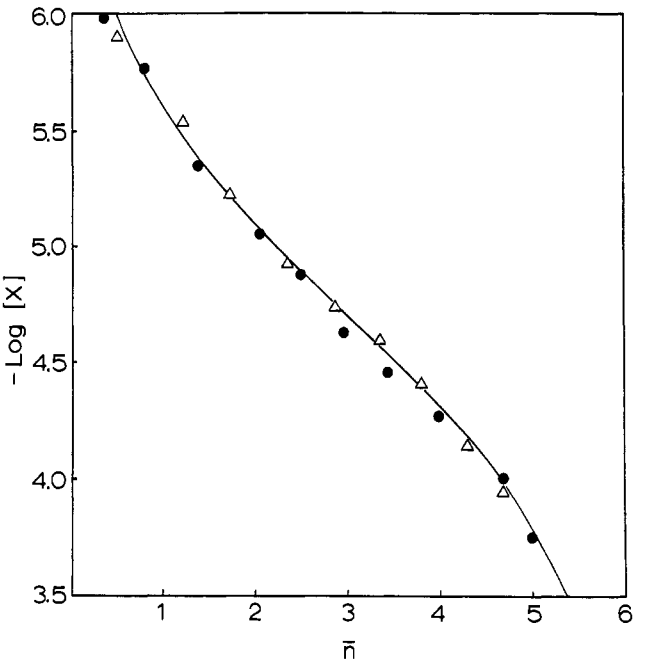

FIGURE 1: Titration of BL-GDH with NADPH. Conditions: 20°, 0.05 M potassium phosphate–10^{-4} M EDTA (pH 7.6). $\bar{n}$ = average number of moles of NADPH bound per 320,000 g of protein; [X] = molar concentration of free NADPH. The BL-GDH concentrations were 0.375 mg/ml (Δ) and 1 mg/ml (●). The smooth curve was calculated for 6 binding sites with a Hill constant of 0.8 and $[X]_{1/2} = 2 \times 10^{-5}$ M.

The circular dichroism of the coenzyme alone was subtracted from the circular dichroism of the GDH–coenzyme mixture (CD_{obsd}). This calculation was based on the following equation for N distinct classes of binding site.

$$CD_{obsd} - (\epsilon_l - \epsilon_r)[X_0] = [X_0]\Sigma f_i[(\epsilon_l - \epsilon_r)_i - (\epsilon_l - \epsilon_r)_o]$$

Where $[X_0]$ is the total coenzyme concentration, $(\epsilon_l - \epsilon_r)_o$ is the characteristic value of $\epsilon_l - \epsilon_r$ for free coenzyme at the wavelength of observation, $(\epsilon_l - \epsilon_r)_i$ is the characteristic value for coenzyme bound to sites in category i, and f_i is the fraction of the total coenzyme bound to sites in category i.

Results

Binding of NADPH. Table I compares the values of F_∞/F_0 for both NADH and NADPH in the presence and absence of GTP. The graphical method used to present the binding studies utilizes plots of − log [X] (where X is the free ligand concentration) *vs.* $\bar{n}$. These plots are used because they reflect both stoichiometry and the nature of the binding equilibrium (Weber, 1965). Calculations of the concentrations of free and bound coenzyme and $\bar{n}$ are based on the equations of Lawrence (1952).

The titration of BL-GDH with NADPH in the absence of GTP is shown in Figure 1. The smooth curve drawn through the points was calculated for 6 binding sites with 50% saturation occurring at a free coenzyme concentration of 20 μM. This value agrees with the dissociation constant determined kinetically (Frieden, 1963b) and fluorometrically (Fisher, 1960; Fisher and McGregor, 1960). The Hill constant (Wyman, 1964) for binding has a value of 0.8, indicative of anticooperative binding. To check for protein concentration dependence, the GDH concentration was varied more than

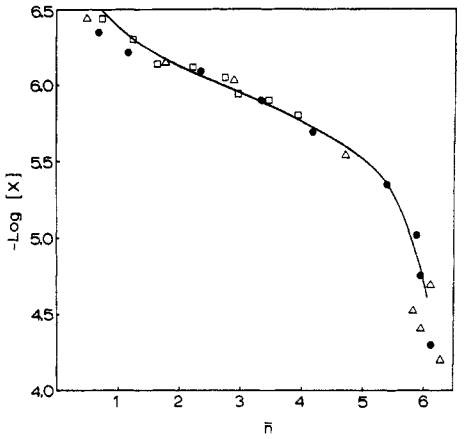

FIGURE 2: Titration of BL-GDH with NADPH in the presence of 300 μM GTP. The GDH concentrations were 0.15 mg/ml (□), 0.375 mg/ml (Δ), and 1 mg/ml (●). The smooth curve was calculated for six binding sites with a Hill constant of 1.5 and $[X]_{1/2} = 10^{-6}$ M. Conditions are given under Figure 1.

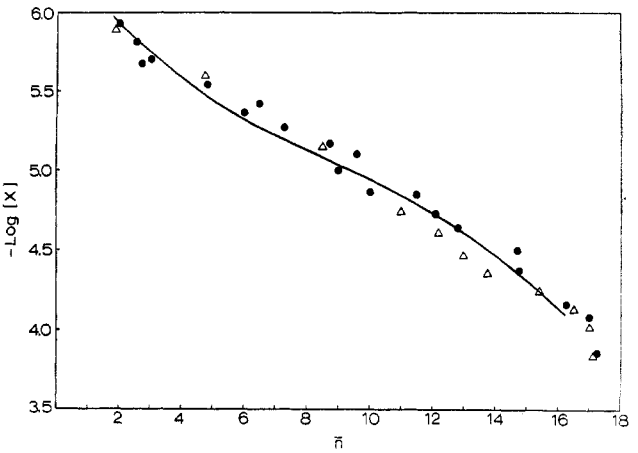

FIGURE 4: Titration of BL-GDH with NADH in the presence of 300 M GTP. The GDH concentrations were 0.375 mg/ml (Δ) and 1 mg/ml (●). The illustrated curve is a smooth curve through the data points and does not represent a theoretical binding curve. Conditions are given under Figure 1.

fifteenfold—from 0.15 to 2.5 mg per ml. The resulting titration curves coincide with the one shown (Figure 1). In the concentration range covered, the weight-average molecular weight varies between 4×10^5 and 2×10^6 (Eisenberg, 1970). Thus the binding of NADPH is independent of the degree of association.

When 300 μM GTP is present, NADPH is more tightly bound (Figure 2). Fifty per cent saturation occurs at a free coenzyme concentration of 1 μM. The titration has a well-defined end point at $\bar{n} = 6$, consistent with the finding that BL-GDH is a hexamer (Eisenberg, 1970). The Hill constant (Wyman, 1964) for the binding of NADPH in the presence of GTP has a value of 1.5, indicative of cooperative binding.

With DF-GDH, there is no fluorescence enhancement of NADPH, either in the presence or absence of GTP. This indicates that NADPH binds very poorly or not at all. Activity measurements are consistent with this conclusion. NADPH had $^1/_{40}$th the activity of NADH.

Binding of NADH to BL-GDH. The titration curve for NADH in the absence of GTP is illustrated in Figure 3. The maximum measured value of $\bar{n}$ is 8 at a free coenzyme concentration of 2×10^{-4} M. The nonintegral number of coenzyme molecules bound must represent additional sites for NADH.

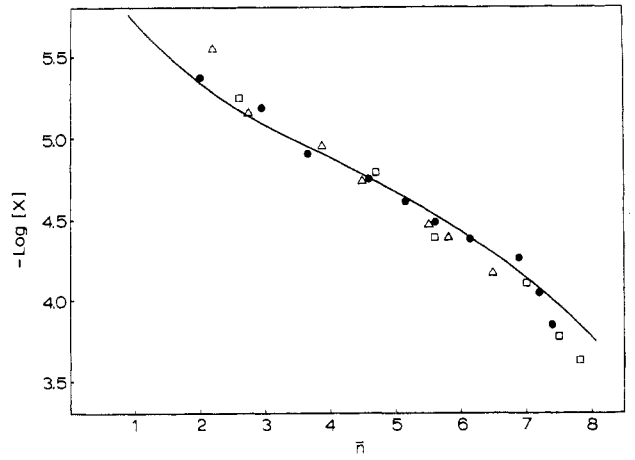

FIGURE 3: Titration of BL-GDH with NADH. The GDH concentrations were 0.2 mg/ml (□), 0.375 mg/ml (Δ), and 1 mg/ml (●). The illustrated curve is a smooth curve through the data points and does not represent a theoretical binding curve. Conditions are given under Figure 1.

The titration curve obtained in the presence of GTP is shown in Figure 4. The striking observation is the large amount of NADH bound—a measurable $\bar{n} = 18$ is obtained. NADH is more strongly bound in the presence of GTP. In this respect the binding is similar to that found for NADPH. Titration of GDH concentrations ranging from 0.2 to 2.5 mg per ml gave results similar to those shown in Figures 3 and 4.

We carried out the following experiments in order to determine whether the value of F_∞/F_0 is applicable at the high values of $\bar{n}$ attained. First we determined the relative quantum yield at various values of $\bar{n}$ ranging from 0.2 to 15. After accounting for incomplete binding, F_∞/F_0 was found to be constant.

The binding was then examined in the ultracentrifuge equipped with scanner (Schachman, 1963). A solution containing 208 μM NADH, 300 μM GTP, and 2 mg/ml of GDH was sedimented at 48,00 rpm at 20° until the boundary was well separated from the meniscus. The absorbancies of the supernatant liquid and of the plateau region measured at 360 nm yielded the proportions of free and bound NADH. The value of $\bar{n}$ obtained was 17.4 ± 2.5. The value of $\bar{n}$ calculated from the fluorescence measurements was 16.7 ± 1.5. Thus the two titration curves and the sedimentation measurements are consistent with additional binding sites for NADH.

Scatchard plots of the NADH binding data show significant nonlinearity. Because of the heterogeneity of NADH binding, the binding constants obtained by any graphical analysis should be interpreted cautiously (Klotz and Hunston, 1971).

Binding NADH to DF-GDH. The titration curves for NADH in the presence and absence of GTP are shown in Figure 5. The results are similar to those obtained with BL-GDH in that a large number of sites (>1/subunit) are titrated in the presence of GTP. The larger experimental error reflects the smaller enhancement factor (Table I), the weaker binding, and the correction for incident light absorption characteristic of DF-GDH.

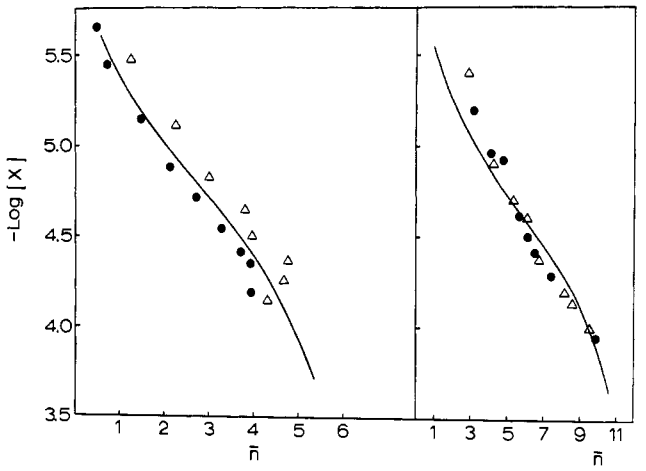

FIGURE 5: Titration of DF-GDH in the absence (left) and presence (right) of 300 μM GTP. The GDH concentrations were 0.375 mg/ml (△) and 1 mg/ml (○). The illustrated curves are smooth curves through the data points and do not represent theoretical binding curves. Conditions are given under Figure 1.

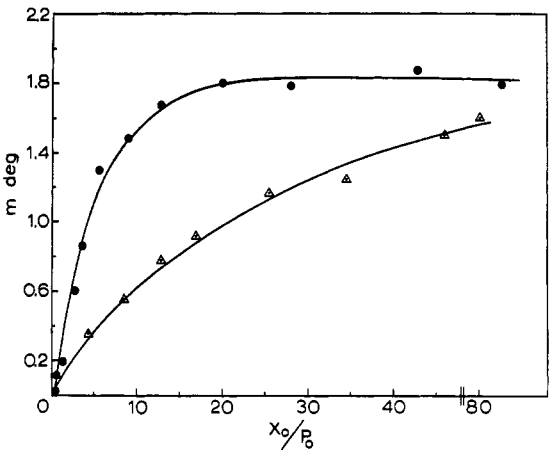

FIGURE 6: Circular dichroism at 335 nm of a mixture containing BL-GDH (0.75 mg/ml) and varying concentrations of NADPH. The circular dichroism of the coenzyme alone was subtracted from the circular dichroism of the GDH–coenzyme mixture; this difference is given in millidegrees (m deg). [X_0] = total ligand concentration, [P_0] = molar concentrations of GDH. (△) No GTP added; (●) 300 μM present. Path length = 1 cm. Other conditions are given under Figure 1.

Binding studies were also carried out using the ultracentrifuge. Again, good correlation was found. An $\bar{n} = 8.1 \pm 1.0$ was obtained from the scanner; an $\bar{n} = 7.6 \pm 0.75$ was obtained from fluorescence data. The concentrations used were 180 μM NADH, 300 μM GTP, and 4 mg/ml of DF-GDH.

Circular dichroism provides useful information on the binding of small molecules to proteins and is especially applicable in the case of GDH, where there is multiple binding (Jallon *et al.*, 1970; Jallon and Iwatsubo, 1971; Koberstein and Sund, 1971). Because of the large protein absorbance below 300 nm, we examined only the 340-nm absorption band of the reduced coenzyme. The maximum absorbance is at 335 nm and the corresponding values of $\epsilon_l - \epsilon_r$ are -0.425 cm^{-1} M^{-1} for free NADH and -0.350 cm^{-1} M^{-1} for free NADPH. The difference between the two coenzymes is reproducible.

Figure 6 illustrates the binding of NADPH in the presence and absence of GTP. The contribution of free coenzyme was subtracted from the circular dichroism as previously described; this contribution was minimal at low values of [X_0]/[P_0], but significant at values of [X_0]/[P_0] greater than 30. The negative band at 335 nm becomes positive upon binding of the coenzyme to the enzyme. In agreement with the fluorescence data, GTP causes stronger binding and allows calculation of a stoichiometric ratio near 6 for NADPH. The binding is weaker in the absence of GTP and does not allow accurate extrapolation of site numbers. However, the plateau reached at high NADPH concentrations is approximately the same in the presence and absence of GTP.

Mixtures of DF-GDH and NADPH give spectra characteristic of free coenzyme. This result is expected since the fluorescence data indicate no binding of NADPH to DF-GDH.

When NADH and BL-GDH are examined in the absence of GTP, the results are similar to those obtained with NADPH (Figure 7). However, the addition of GTP causes the band centered at 335 nm to be *positive* at low concentrations of NADH and *negative* at high concentrations of NADH (Figure 7). This suggests that the change of sign is correlated with binding of additional NADH (Jallon and Iwatsubo, 1971; Koberstein and Sund, 1971).

The sign reversal in the presence of GTP may mean that the intrinsic circular dichroism of NADH bound to the secondary sites is very negative. An alternative explanation is that the 340-nm transition of these additional sites is coupled with another strong electric or magnetic moment that is in close proximity and properly oriented. Bands of strong rotational strength would result from the coupling (Urry, 1970). A number of higher energy transitions are available, including those of the purine rings of the GTP and the other NADH–NADPH molecules.

The addition of either AMP, ADP, or ATP in equimolar concentration with GTP causes immediate reversal of the negative band and a return to the positive band shown in the absence of GTP. This is consistent with other findings (Cross and Fisher, 1970; Frieden and Coleman, 1967) which show that the binding of the adenine nucleotides excludes the guanine nucleotides.

High concentrations (1 mM) of either NAD^+ or $NADP^+$ do not reverse the strong negative band at 335 nm. This implies that the binding of the oxidized coenzyme at these additional sites is weak or nonexistent. Analog studies using re-

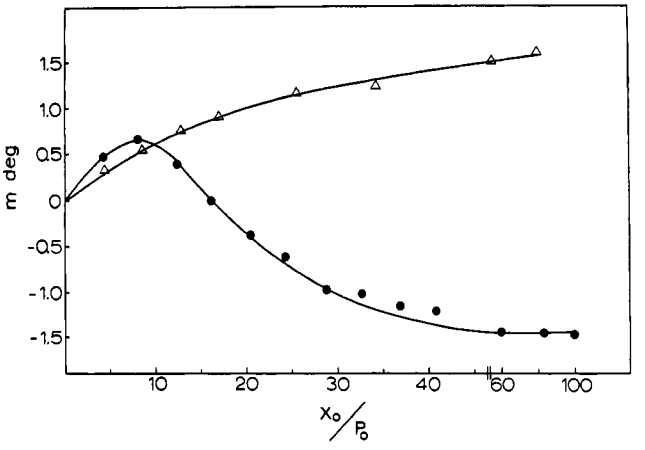

FIGURE 7: Circular dichroism at 335 nm of a mixture containing BL-GDH (0.75 mg/ml) and varying concentrations of NADH. (△) No GTP added; (●) 300 μM GTP present. Refer to Figures 1 and 6 for conditions and abbreviations.

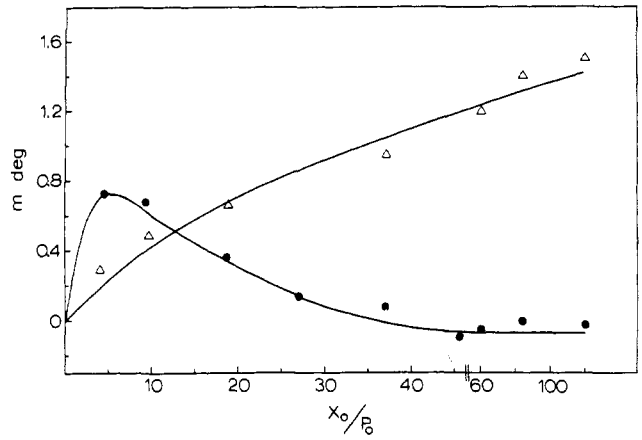

FIGURE 8: Circular dichroism at 335 nm of a mixture containing DF-GDH (0.75 mg/ml) and varying concentrations of NADH. (Δ) No GTP added; (●) 300 μM GTP present. Refer to Figures 1 and 6 for conditions and abbreviations.

duced 3-acetylpyridine-adenine dinucleotide and reduced nicotinamide-hypoxanthine dinucleotide confirm the specificity of the extra NADH sites. Neither analog shows a negative band in the presence of GTP and BL-GDH.

Results obtained with DF-GDH and NADH are shown in Figure 8. In the absence of GTP, the bound NADH has the same sign and rotational strength found with BL-GDH. The addition of GTP causes the rotational strength at 335 nm to disappear entirely at high concentrations of NADH. This is in contrast to BL-GDH, which gives a strong negative band under similar conditions. Since approximately the same numbers of NADH molecules are bound to the two enzymes, the circular dichroism spectra must reflect intrinsic differences between the secondary binding sites.

Formation of the abortive ternary complex with BL-GDH, glutamate, and coenzyme also is useful in stoichiometry determinations. In the absence of GTP, the abortive complex with either NADH or NADPH gives a negative circular dichroism band centered at 335 nm. Figure 9 shows the results for NADH in two cases. In the first, the enzyme concentration is held constant and the coenzyme concentration varied; in the second, the coenzyme concentration is held constant while the enzyme concentration is varied. Stoichiometry calculations from these data give $\bar{n} = 5.4 \pm 0.75$ at saturating levels of coenzyme. Experiments with NADPH

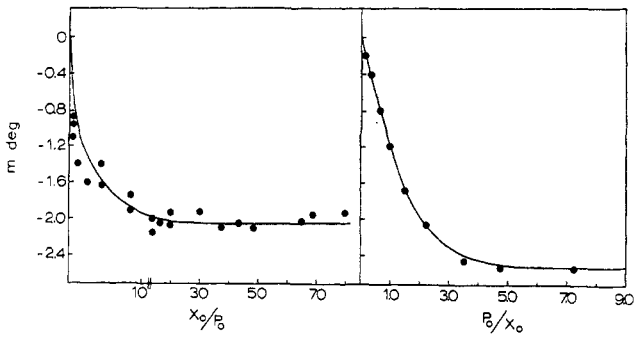

FIGURE 9: Circular dichroism at 335 nm of a mixture containing 0.75 mg/ml of BL-GDH, 10 mM glutamate, and varying concentrations of NADH (left) and of a mixture containing 15 μM NADH, 10 mM glutamate, and varying amounts of BL-GDH (right). Refer to Figures 1 and 6 for conditions and abbreviations.

give the same results. These sites probably represent those that are catalytically functional.

Addition of GTP to the saturated BL-GDH-glutamate-NADH complex causes the appearance of a still stronger negative band. The rotational strength at 335 nm is proportional to the enzyme concentration and is consistent with an increased amount of NADH binding upon GTP addition. The addition of GTP to the saturated BL-GDH-glutamate-NADPH has no effect on the circular dichroism. This agrees with the other fluorescence and circular dichroism data indicating distinct differences between NADH and NADPH binding.

Discussion and Conclusions

NADPH Binding. The binding of NADPH to BL-GDH represents a simple case of stoichiometry. The maximum number bound for NADPH, both in the presence and absence of GTP, is consistent with the hexamer hypothesis for the oligomeric molecule (Eisenberg, 1970). In this way BL-GDH is similar to the other dehydrogenases where the molecular weights of the polypeptide chains closely approximate the equilvalent weights of the coenzyme binding sites. However, the apparent binding order of NADPH to BL-GDH changes from negative interaction ($j = 0.8$) in the absence of GTP to cooperativity ($j = 1.5$) in the presence of GTP.

ADP and GTP alter the apparent affinity of the enzyme for NADPH. ADP, by binding to subsites I and III in the terminology of Cross and Fisher (1970), competes with the coenzyme for common binding sites. However, GTP addition enhances the binding of NADPH. Thus the activating and inhibitory effects of ADP and GTP, respectively, together with their effects on NADPH binding, suggest that the dissociation of the binary complex (enzyme-reduced coenzyme) is the rate-limiting step of the reaction (Pantaloni and Iwatsubo, 1967). The DF-GDH is NADH specific since it is unable to bind NADPH effectively.

NADH Binding. The binding of NADH to both BL-GDH and DF-GDH is complex. The nonintegral number of binding sites titrated in the absence of GTP and the large number of sites occupied in the presence of GTP imply heterogeneity in NADH binding. One set of these sites is probably the same set (6) involved in NADPH binding. An independent class of sites (12 or more) also binds NADH and is affected by GTP. In the absence of GTP, binding at the secondary sites is weak. Thus only a limited number are titrated within the NADH concentration range covered (Figure 3). In the presence of GTP, the binding is stronger and a larger number of sites are titrated at equivalent NADH concentrations (Figure 4). Thus the actual number of NADH sites is probably the same in the two cases.

The function of the extra NADH sites is uncertain. They may function in activation, but this effect is present only at very high concentrations of coenzyme.

A more interesting possibility is buffering of the NADH concentration in the mitochondrial matrix. Krebs and Veech (1970) have estimated the $NAD^+/NADH$ ratio in the mitochondrion to be approximately 10 for the redox state of perfused rat liver cells. The estimate of mitochondrial NADH binding sites is 160 nmoles/g of rat liver. The concentrations of the dehydrogenases present indicate that GDH could bind about 60 nmoles, assuming 6 binding sites; malate dehydrogenase, 5 nmoles; and NAD^+-specific isocitrate dehydrogenase, 3 nmoles (Bücher, 1970). However, the high NADPH level would effectively compete for the catalytically functional

sites in GDH. Maintenance of the redox potential would necessitate the binding of NADH by other protein molecules. The extra NADH binding sites of GDH could perform this function, especially if substantial levels of GTP were present. Even if the NADPH sites were completely saturated, the other NADH-specific sites would be available to maintain the redox equilibrium.

Acknowledgments

We thank Professor K. E. Van Holde for the use of the circular dichroism spectrophotometer and Mr. R. L. Howard for the amino acid analysis.

References

Anderson, S. R., and Weber, G. (1965), *Biochemistry 4*, 1948.
Babul, J., and Stellwagen, E. (1969), *Anal. Biochem. 28*, 216.
Bücher, T. (1970), *in* Pyridine Nucleotide. Dependent Dehydrogenases, Sund, H., Ed., New York, N. Y., Springer-Verlag.
Cassman, M., and Schachman, H. (1971), *Biochemistry 10*, 1015.
Corman, L., Prescott, L. M., and Kaplan, N. O. (1967), *J. Biol. Chem. 242*, 1383.
Cross, D. G., and Fisher, H. F. (1970), *J. Biol. Chem. 245*, 2612.
Eisenberg, H. (1970), *in* Pyridine Nucleotide Dependent Dehydrogenases, Sund, H., Ed., New York, N. Y., Springer-Verlag.
Eisenberg, H., and Tomkins, G. M. (1968), *J. Mol. Biol. 31*, 37.
Fisher, H. F. (1960), *J. Biol. Chem. 235*, 1830.
Fisher, H. F., and McGregor, L. L. (1960), *Biochim. Biophys. Acta 43*, 557.
Frieden, C. (1963a), *Enzymes 7*, 3.
Frieden, C. (1963b), *J. Biol. Chem. 238*, 146.
Frieden, C. (1964), Subunit Structure of Proteins Biochemical and Genetic Aspects, U. S. Department of Commerce, Springfield, Va.
Frieden, C. (1970), *in* Mechanism of Action of Dehydrogenases, Schwert, G. W., and Winer, A. D., Ed., Lexington, Ky., University of Kentucky Press.
Frieden, C., and Coleman, R. (1967), *J. Biol. Chem. 242*, 1705.
Jallon, J. M., DiFranco, A., and Iwatsubo, M. (1970), *Eur. J. Biochem. 13*, 428.
Jallon, J. M., and Iwatsubo, M. (1971), *Biochem. Biophys. Res. Commun. 45*, 964.
Klotz, I. M., and Hunston, D. L. (1971), *Biochemistry 10*, 3065.
Koberstein, R., and Sund, H. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 19*, 194.
Krebs, H. A., and Veech, R. L. (1970), *in* Pyridine Nucleotide Dependent Dehydrogenases, Sund, H., Ed., New York, N. Y., Springer-Verlag.
Lawrence, D. J. R. (1952), *Biochem. J. 51*, 1968.
Pantaloni, D., and Iwatsubo, M. (1967), *Biochim. Biophys. Acta 132*, 217.
Schachman, H. K. (1963), *Biochemistry 2*, 887.
Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724.
Strecker, H. J. (1955), *Methods Enzymol. 2*, 220.
Sund, H. (1968), *in* Biological Oxidations, Singer, T. P., Ed., New York, N. Y., Interscience.
Weber, G. (1965), *in* Molecular Biophysics, Pullman, B., and Weissbluth, M., Ed., New York, N. Y., Academic Press.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.
Urry, D. W. (1970), *in* Spectroscopic Approaches to Biomolecular Conformation, Urry, D. W., Ed., Chicago, Ill., American Medical Association.