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STUDIES OF COENZYME BINDING TO RABBIT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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Summary

The binding of NAD^+, NADH and adenosine diphosphoribose (Ado-*PP*-Rib) to a stable, highly active and nucleotide-free preparation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate : NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) has been studied. All three nucleotides quench the protein fluorescence to the same extent when they bind to the enzyme, and this property has been used to measure the dissociation constants for the two high-affinity binding sites for the nucleotides. The results indicate negative interactions between, or non-identity of, these two binding sites, to which NAD^+ and NADH bind with similar affinity.

The binding of NAD^+ to the enzyme has been studied by spectrophotometric titrations at 360 nm. It appears that the binding of NAD^+ to each of the four subunits of the enzyme contributes equally to the intensity of this 'Racker' band. The dissociation constants associated with the binding of the third and fourth molecules of NAD^+ estimated from such titrations confirm some previous estimates.

The binding of NADH to the enzyme causes a decrease of intensity of the absorbance of the coenzyme at 340 nm, and the dissociation constants for binding of the third and fourth molecules of NADH have been estimated from spectrophotometric titrations. They are the same as those for NAD^+.

Judging by the apparent dissociation constants, negative interactions on binding the third molecule of NAD^+ or NADH are more marked than those associated with the binding of the second and fourth molecules, suggesting that a major conformational change occurs at half-saturation of the tetramer with coenzyme.

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Introduction

The binding of either NAD^+ or NADH to the tetrameric rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate : NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) has been shown to involve more than one apparent dissociation constant [1]. This would indicate either that the four subunits in the oligomer are not functionally identical, or that the binding of the coenzyme to the enzyme involves negative co-operativity.

Binding studies using direct separation methods such as equilibrium dialysis [2] or ultracentrifugation [3,4] have clearly demonstrated the existence of four binding sites per tetramer for NAD^+ or NADH. However, previous studies of coenzyme binding have demonstrated the existence of three dissociation constants for NAD^+ or NADH. The high affinity of two out of the four sites in the tetramer has precluded direct estimation of dissociation constants for the first two molecules of coenzyme to bind to the oligomer, and it has generally been reported that the dissociation constants for the first two sites were "smaller than 0.1 μM" [3].

Studies of NAD^+ binding [4,5] utilizing the 'Racker' absorption band [6] at 360 nm, formed when NAD^+ binds to the enzyme, or the quenching of protein fluorescence [7] concomitant with coenzyme binding, have indicated that the fourth equivalent of NAD^+ to bind to the enzyme may not produce the same change in spectroscopic parameter as is associated with the binding of the first three molecules. Similar results have been reported for the changes in ellipticity of 335 nm that accompany NADH binding to apoenzyme [4].

Despite the apparent chemical identity of the polypeptide chains in the tetramer [8], such results raise the possibility that there may be small chemical differences between the polypeptide chains, or that identical polypeptide chains are arranged in a spatially asymmetric manner. However, many of the studies referred to above have used enzyme with a significantly lower [3,4] specific activity than the highest value reported in the literature [9]. Further complications may have arisen from the instability of the apoenzyme that has been noted in some studies.

In view of the possible significance of the apparent non-equivalence of the coenzyme binding sites, the binding of NAD^+, using the Racker absorption band, and of NADH, using the hypochromicity of the reduced nicotinamide absorption band that occurs on binding, has been studied using freshly prepared apoenzyme with a specific activity slightly higher than any previously reported. In addition, by making use of the great sensitivity of fluorometric techniques, the binding of both coenzymes to the first two binding sites per oligomer has been studied, and the first direct estimates of K_1 and K_2 obtained.

Materials and Methods

Crystalline rabbit muscle glyceraldehyde-3-phosphate dehydrogenase holoenzyme was isolated from the back and hind leg muscles of freshly killed rabbits. The procedure described by Ferdinand [9] was followed. The final

specific activity of the product after several recrystallizations to remove haem was 200 μmol NADH produced/min per mg enzyme, somewhat higher than the value obtained by Ferdinand.

Apoenzyme was prepared from holoenzyme by treatment with activated charcoal, using a batchwise procedure. Holoenzyme crystals were dissolved in 0.1 M triethanolamine buffer, pH 6.7 containing 1 mM EDTA, and thoroughly dialysed against the same buffer to remove all residual ammonium sulfate. After dialysis the enzyme solution was made 1 mM with respect to dithiothreitol, and activated charcoal, prepared as described by Krimsky and Racker [10], was added to give a weight/weight ratio of about two or three to one, charcoal to protein. The solution was thoroughly mixed, and incubated at 0—4°C for 45—50 min with frequent mixing. The charcoal was removed by filtration through a millipore filter, and the enzyme solution dialysed against triethanolamine buffer, pH 7.5, prior to characterization or use.

The apoenzyme contained no bound nucleotide, as judged by enzymatic analysis for NAD^+ and NADH, the ratio of absorbancies at 280 and 260 nm (1.95) or the absorbance at 260 nm of the supernatent obtained after acid precipitation of the protein by the addition of trichloroacetic acid. The apoenzyme had the same specific activity as the holoenzyme and was stable for at least 36 h at room temperature.

Protein concentrations were estimated by absorbance measurements using extinction coefficients at 280 nm of 0.98 $cm^2 \cdot mg^{-1}$ for holoenzyme and 0.83 $cm^2 \cdot mg^{-1}$ for apoenzyme [11]. A molecular weight for the tetramer of 140 000 [12] was used throughout. Activity measurements were made in 0.1 M triethanolamine buffer, pH 9.0, containing 1 mM EDTA with 50 mM phosphate, 2 mM DL-glyceraldehyde 3-phosphate and 1 mM NAD^+. Absorbance measurements were made using 1 cm light-path quartz cuvettes, with either a Cary, model 14, or a Unicam SP500 spectrophotometer for absorbance titrations, or a Unicam SP 1800 spectrophotometer for spectral measurements. All absorbance measurements were corrected for the appropriate cell and solution blanks. Fluorescence measurements were made with a Farrand Mark I fluorimeter. Fluorescence measurements were corrected for blanks, and for absorbance of incident light by added nucleotides.

NAD^+ (grade 1) and triethanolamine hydrochloride were obtained from Boehringer. NADH (grade III) and dithiothreitol were obtained from Sigma. Ado-*PP*-Rib was from P-L Biochemicals. Other reagents were the highest grade commercially available. All solutions were made up with double glass-distilled water, and except for the enzyme solutions, were filtered through sintered glass prior to use.

Results

Spectrophotometric titrations

An extrinsic absorption band centered at 360 nm is formed when NAD^+ binds to the apoenzyme. A titration of the absorbance at 360 nm was performed with an apoenzyme concentration of 29.4 μM enzyme and NAD^+ concentrations up to 500 μM. The results (Fig. 1) show that up to a coenzyme/enzyme ratio of 2, a linear increase of absorbance occurs, indicating that up to

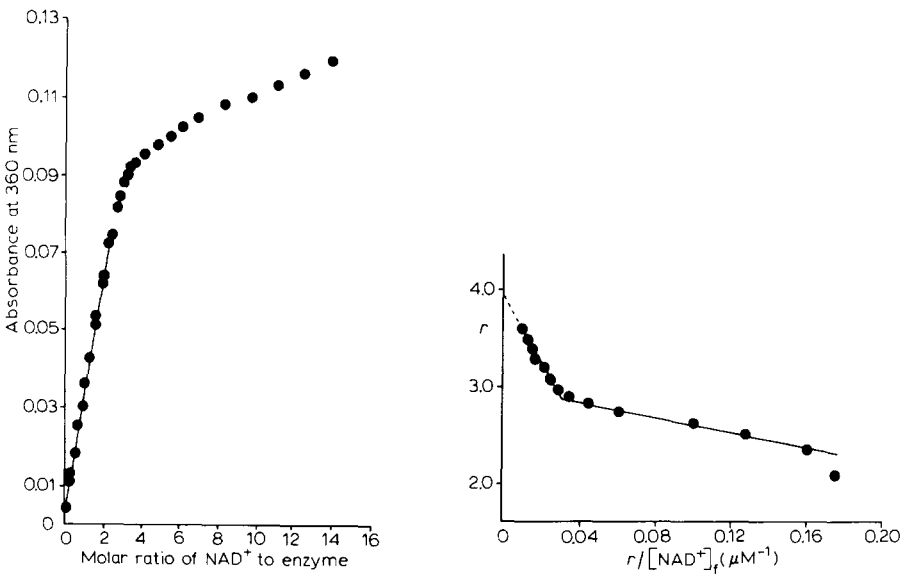

Fig. 1. Spectrophotometric titration of the apoenzyme with NAD^+ at 360 nm. The enzyme concentration was 29.4 μM in 0.1 M triethanolamine buffer, pH 7.6, containing 1 mM EDTA. The temperature was 25° C.

Fig. 2. Scatchard plot for the binding of NAD^+ to the enzyme. The data were calculated from the results of the titration shown in Fig. 1, using an extinction coefficient of 1.05 $mM^{-1} \cdot cm^{-1}$ for bound NAD^+, estimated as described in the text. The ratio, r, of the concentration of bound nucleotide to the total enzyme concentration, is plotted against $r/[NAD^+]_f$, where $[NAD^+]_f$ is the concentration of unbound nucleotide.

this point all of the NAD^+ added is bound to the enzyme. A millimolar extinction coefficient of 1.05 cm^{-1} for bound NAD^+ is calculated from the slope. From the remainder of the titration, the amount of NAD^+ bound at any point can be calculated using this extinction coefficient if it is assumed that all four sites in the enzyme tetramer contribute equally to the absorbance at 360 nm. A Scatchard plot [13] of the data above half-saturation is shown in Fig. 2. The biphasic nature of the plot indicates either the existence of non-identical sites, or negative homotropic cooperativity between sites 2, 3 and 4. Apparent dissociation constants for the third and fourth molecules of NAD^+ bound, calculated from the slopes of the two linear sections in Fig. 2, are 4 μM and 36 μM, respectively.

Difference spectra for enzyme-NADH complexes (E-NADH minus E + NADH) recorded in a double-beam spectrophotometer indicated that a negative difference, centered at 335 nm, was produced. Titrations of the difference in absorbance at 335 nm between free NADH and NADH in the presence of 24.2 μM enzyme were carried out. The results (Fig. 3) show that the magnitude of the difference increased linearly with the concentration of added coenzyme until a coenzyme/enzyme ratio of 2 was reached, indicating that up to this point all of the NADH was bound. From the slope of this line, a millimolar extinction coefficient at 335 nm of 4.7 cm^{-1} is obtained for enzyme-bound NADH. Using the difference between the extinction coefficients for bound and free NADH, the amount of NADH bound at any subsequent point in the

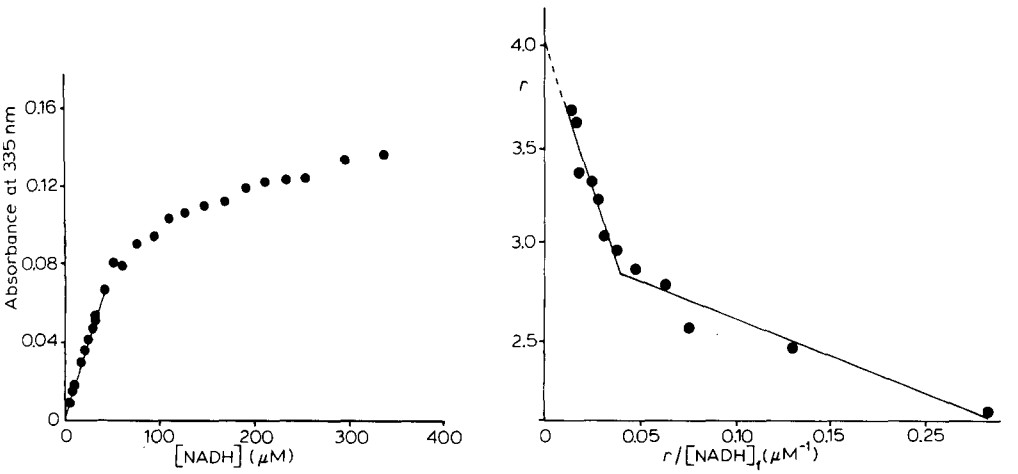

Fig. 3. Spectrophotometric titration of 24.2 μM enzyme with NADH at 335 nm. Other conditions were as described in the legend to Fig. 1.

Fig. 4. Scatchard plot for the binding of NADH to the enzyme. The data were calculated from the titration results shown in Fig. 3, using an extinction coefficient of 4.7 $mM^{-1} \cdot cm^{-1}$ for bound NADH. r is defined in the legend to Fig. 2.

titration can be calculated, if it is assumed that the binding of each molecule of NADH to the tetramer produces an equal change in the extinction coefficient of NADH. A Scatchard plot (Fig. 4) shows the results of such calculations for the data above half-saturation. As with NAD^+, the data cannot be described by a single dissociation constant, indicating either the presence of non-identical binding sites or negative homotropic cooperativity in NADH binding. Apparent dissociation constants of 4 μM and 35 μM are obtained for the third and fourth molecules of NADH to bind to the tetramer.

The absorption spectrum of bound NADH was obtained by recording the spectrum of 29.7 μM NADH in the presence of 37.9 μM enzyme tetramers, conditions under which all of the coenzyme is bound to the enzyme. The results (Fig. 5) show a 26% hypochromicity and a 4—5 nm red-shift in the absorption maximum of NADH on binding to glyceraldehyde-3-phosphate dehydrogenase.

Protein fluorescence quenching titrations

Coenzyme binding has also been studied by making use of the quenching of the protein fluorescence that occurs when NAD^+ or NADH binds to the enzyme. In experiments with 9.9 μM enzyme tetramers, the protein fluorescence, excited at 295 nm and emitted at 350 nm, was quenched by the addition of NAD^+, NADH or Ado-*PP*-Rib. The results of such titrations are shown in Fig. 6. With each of the nucleotides used, the quenching was a linear function of the nucleotide concentration up to a nucleotide/enzyme ratio of 2, indicating that under these conditions all of the nucleotide was bound to the enzyme. From the data, the quenching coefficient, Q, may be obtained from the relationship:

$$Q = \frac{\text{Fluorescence of 1 } \mu\text{M enzyme with 1 } \mu\text{M bound } NAD^+}{\text{Fluorescence of 1 } \mu\text{M free enzyme}}$$

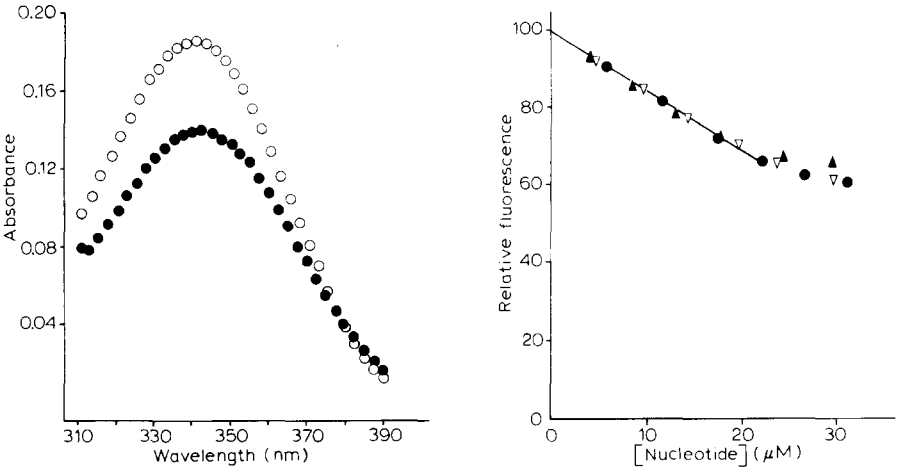

Fig. 5. Absorption spectra of free (○) and enzyme-bound (●) NADH. The concentration of NADH was 29.7 μM in 0.1 M triethanolamine buffer, pH 7.6. For the spectrum of enzyme-bound NADH, the solution also contained 37.9 μM enzyme.

Fig. 6. Quenching of the protein fluorescence of the apoenzyme by nucleotides. The fluorescence of 9.9 μM enzyme in 0.1 M triethanolamine buffer, pH 7.6, excited at 295 nm and measured at 350 nm, was measured with various concentrations of NAD^+ (○), NADH (▽) and ADPR (▲). The fluorescence is shown in arbitrary units assuming that the fluorescence of the apoenzyme is 100 (temperature, 25° C).

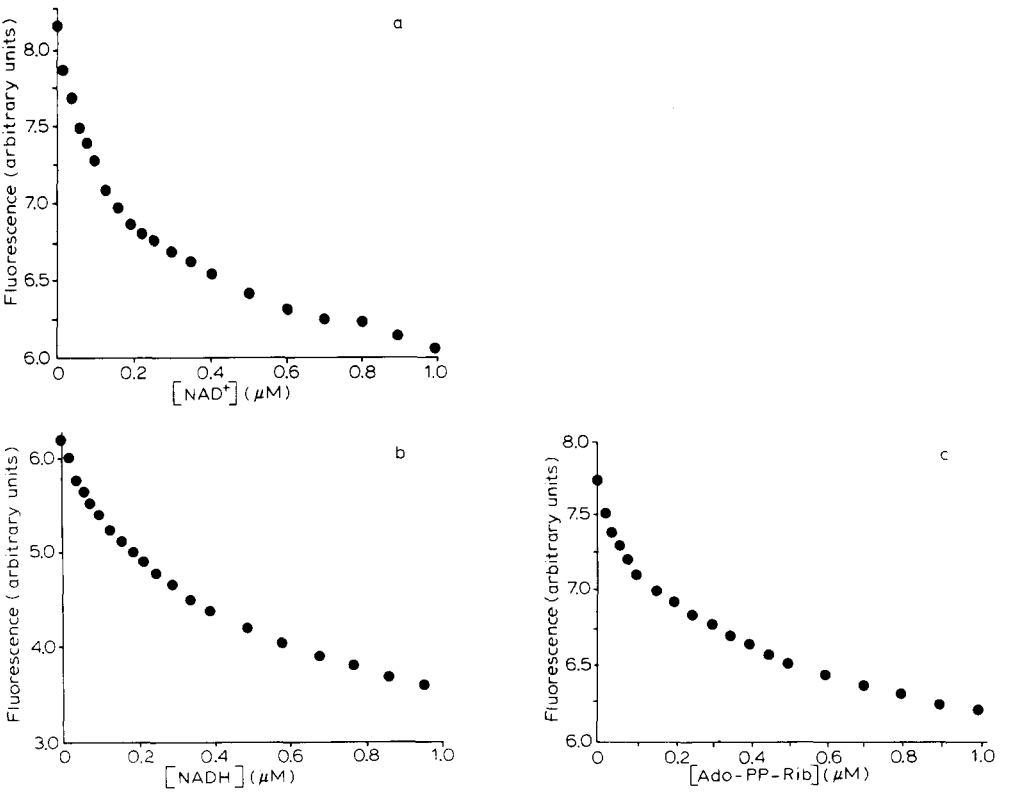

Fig. 7. Protein fluorescence titrations of apoenzyme with nucleotides. (a) 0.104 μM enzyme titrated with NAD^+. (b) 0.108 μM enzyme titrated with NADH. (c) 0.099 μM enzyme titrated with Ado-*PP*-Rib. Other conditions were as for Fig. 6.

Within the experimental error, each of the nucleotides gave the same value for Q, 0.85. The linearity of the data up to half-saturation of the tetramer indicates that at least the first two binding sites contribute equally to the protein fluorescence quenching. The calculation of binding data from protein fluorescence quenching usually depends on saturating the protein with ligand to estimate the maximal quenching of the fluorescence. By making use of the stoichiometric binding of nucleotide to the first two sites in the tetramer at high enzyme concentrations, the 'per site' quenching of fluorescence can be calculated for NAD^+, NADH and Ado-*PP*-Rib. Binding data can then be calculated without experimentally determining the maximal quenching.

Because of the great sensitivity attainable in fluorescence measurements, and the relatively high quantum yield of protein fluorescence, it is possible to use a small enough enzyme concentration to titrate the first two sites in the tetramer. Such titrations using protein concentrations of about 0.1 μM were carried out with NAD^+, NADH and Ado-*PP*-Rib (Fig. 7). Using the quenching coefficients obtained in the previous experiment, the amount of nucleotide bound at each point in the titration can be calculated if it is assumed that each

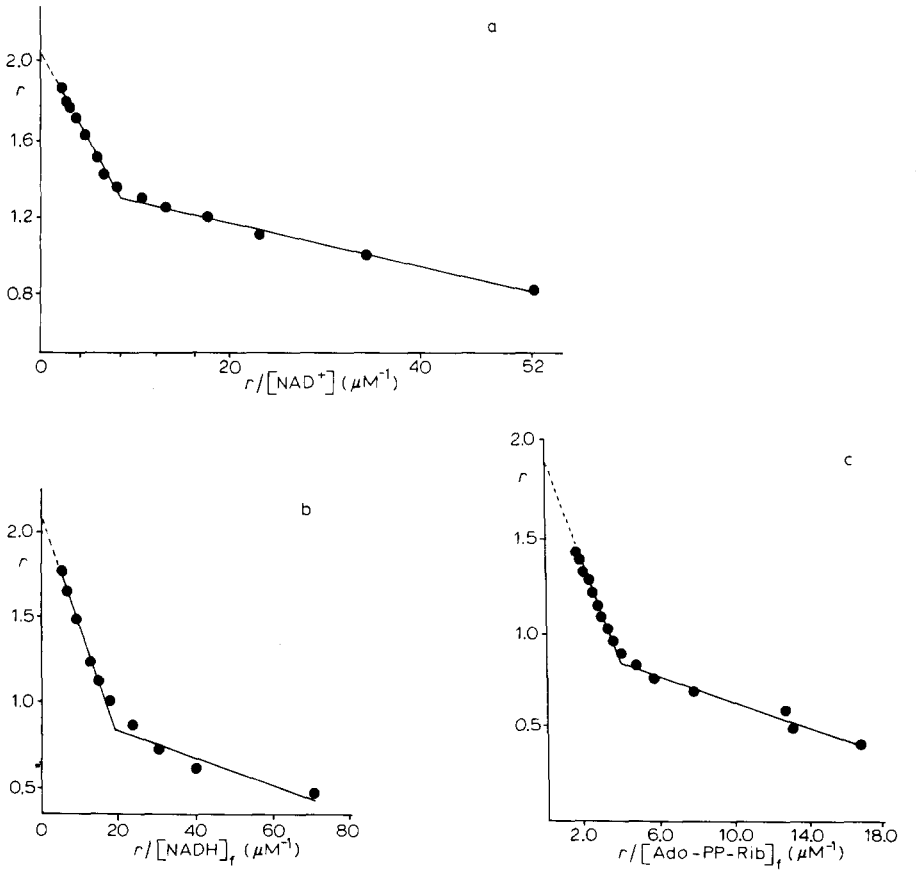

Fig. 8. Scatchard plots for the binding of nucleotides to the enzyme. The data were calculated from the results shown in Fig. 7 as described in the text. (a) NAD^+ binding, (b) NADH binding and (c) Ado-*PP*-Rib binding. r is defined in the legend to Fig. 2.

TABLE I

APPARENT DISSOCIATION CONSTANTS FOR ENZYME COMPLEXES WITH NAD^+, NADH AND Ado-*PP*-Rib

The dissociation constants K_1 and K_2 for binding at the first two sites of the tetramer were estimated from measurements of protein fluorescence quenching (Fig. 8) and those for the other two sites, K_3 and K_4, from spectrophotometric titrations (Figs 2 and 4) as described in the text. All the measurements were made in 0.1 M triethanolamine buffer, pH 7.6, at 25°C.

Ligand	Apparent dissociation constant (μM)			
	K_1	K_2	K_3	K_4
NAD^+	0.01	0.09	4.0	36
NADH	0.008	0.06	4.0	35
Ado-*PP*-Rib	0.035	0.262		

subunit in the tetramer makes an equal contribution to the fluorescence of the protein, and that each subunit has an identical value for Q. The results of such calculations are shown in Fig. 8 in the form of Scatchard plots. For each of the nucleotides, the data requires at least two apparent dissociation constants indicating either the existence of non-identical binding sites, or negative homotropic cooperativity in nucleotide binding to the first two sites in the tetramer.

The apparent dissociation constants for the first two sites to be saturated by these nucleotides are shown in Table I, together with the apparent dissociation constants, obtained from the spectrophotometric titrations, for NAD^+ and NADH binding to the third and fourth sites in the tetramer.

Discussion

In the present work it has been possible to make the first direct determinations of the dissociation constants for nucleotides binding at the first two sites per tetramer, by making use of the quenching of protein fluorescence that occurs on coenzyme binding. The results with NAD^+, NADH and Ado-*PP*-Rib indicate that the binding of these nucleotides to the first two sites in the tetramer produces an equal quenching of the protein fluorescence.

The apparent dissociation constants for the binding of NAD^+ or NADH to these two high-affinity sites are 0.01 μM and 0.09 μM for NAD^+, and 0.008 μM and 0.06 μM for NADH. In contrast to most dehydrogenases, glyceraldehyde-3-phosphate dehydrogenase evidently has similar affinities for NAD^+ and NADH. The binding of the first two molecules of coenzyme to the tetramer clearly shows behaviour consistent with either negative homotropic cooperativity or the existence of intrinsically non-equivalent binding sites.

The protein fluorescence quenching coefficients obtained in this work agree well with that reported previously for NAD^+ binding to the two lower affinity sites [14]. This suggests that there is an equal contribution to the quenching by ligand binding to each of the four coenzyme binding sites in the oligomer.

Each of the three nucleotides used in this study produce the same quenching of the protein fluorescence on binding. This suggests that the quenching may be due to either direct interaction of the adenine moiety of the coenzyme

with one or more fluorescent residues in the enzyme, or to an overall conformational change, rather than to energy transfer from the protein to the coenzyme. In the complexes E-NAD^+ and E-NADH, energy transfer from the protein to the coenzyme is possible; however, in the E-Ado-*PP*-Rib complex, which has no absorption bands in the region 300–450 nm, such energy transfer is not possible.

The values of the apparent dissociation constants, directly determined in this work, for NAD^+ binding to the first two sites per tetramer differ somewhat from those indirectly estimated by Conway and Koshland [2]. In an equilibrium dialysis experiment, these workers found that less than 5% of the NAD^+ was removed from E-NAD_2 by prolonged dialysis. From this experiment they estimated a very approximate value of the dissociation constant of the second NAD^+ molecule to bind of about 10^{-9} M. To account for the sharp end-point in studies of the effects of NAD^+ on SH-group reactivity, they inferred a value for K_1 of 10^{-11} M from their approximate estimate of K_2.

In the spectrophotometric titrations with NAD^+ or NADH, the Scatchard plots of the data extrapolate to four binding sites per tetramer. The extrapolation of the data in either case is not large as approximately 95% saturation of the tetramer is achieved; that is, the fourth site is 80% saturated with coenzyme. This extrapolation to four sites per tetramer supports the assumption that is made, that each site contributes about equally to the 'Racker' band in E-NAD^+, and to the hypochromicity of the absorption of the reduced nicotinamide moiety in E-NADH. The apparent dissociation constants estimated in this work for NAD^+ and NADH binding to the two sites with lower affinity are in excellent agreement with recent determinations using direct separation techniques [15], which do not depend on the assumptions that must be made using spectroscopic techniques. This adds further support to the conclusion that each molecule of coenzyme bound contributes about equally to the fluorescence or absorbance changes.

It has previously been reported that enzyme-bound NADH had the same spectral characteristics as free NADH [16], although a more recent study has indicated an 18% hypochromicity of NADH absorption on combination with the rabbit muscle enzyme [17]. The negative difference spectrum reported here results from a 24% hypochromicity and a 4–5 nm red shift in the absorption maximum of the nicotinamide ring of the bound NADH. These absorption changes probably reflect the same optical interaction that produces an ellipticity band at 335 nm [4].

The apparent dissociation constants for each of the four coenzyme binding sites in the tetramer, shown in Table I, clearly indicate either that there is negative homotropic cooperativity between all four sites in the tetramer, or that the sites are in some way non-equivalent. It is of interest that the ratios between the dissociation constants for sites one and two for each of the nucleotides studied in this work, and the ratio between the dissociation constants for sites three and four for NAD^+ and NADH, are approximately the same. There is, however, a much larger change in affinity between sites two and three for NAD^+ and NADH, some 40-fold compared with an approximately 9-fold change change between sites one and two or sites three and four. This suggests that half-saturation of the tetramer produces a major conformational change in

addition to the sequential conformational changes involved in negative cooperativity. Alternatively, there may be some inherent asymmetry in the tetramer such that the four subunits are related in dimers. The recent X-ray crystallographic studies [18] of the lobster-muscle enzyme show that the coenzyme binding sites are closely related across one of the three two-fold axes in the tetramer. In such a case it may be that coenzyme binding to the second subunit is weaker than binding to the first subunit due to steric or electrostatic effects. Half-saturation of the tetramer produces a conformational change in the remaining two subunits causing a much greater decrease in affinity. Coenzyme binding to the remaining two sites is then subject to the same steric or electrostatic factors as the first two sites.

Acknowledgements

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