Cooperativity and Noncooperativity in the Binding of NAD Analogues to Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: Using NAD analogues as ligands, the structural requirements for negative cooperativity in binding to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase were examined. Although the affinity of nicotinamide hypoxanthine dinucleotide is considerably lower than that of NAD^+, it also binds to the enzyme with negative cooperativity. Two pairs of nicotinamide hypoxanthine dinucleotide binding sites were distinguished, one pair having an affinity for the analogue which is 15 times that of the second pair. Negative cooperativity is also found in the K_m values for the analogue. Thus modification of the adenine ring of NAD^+ to hypoxanthine does not abolish negative cooperativity in coenzyme binding. Adenosine diphosphoribose binding to the same enzyme shows neither positive nor negative cooperativity, indicating that cooperativity apparently requires an intact nicotinamide ring in the coenzyme structure, under the conditions of these experiments. Occupancy of the nicotinamide subsite of the coenzyme binding site is not necessary for half-of-sites reactivity of alkylating or acylating compounds (Levitzki, A. (1974), *J. Mol. Biol. 90,* 451–458). However, it can be important in the negative cooperativity in ligand binding, as illustrated by adenosine diphosphoribose which fails to exhibit negative cooperativity. Occupancy of the adenine subsite by adenine is important for stabilization of the enzyme against thermal denaturation. Whether the stabilization is due to an altered conformation of the subunits or stabilization of the preexisting structure of the apoenzyme cannot be determined from these studies. However, nicotinamide hypoxanthine dinucleotide does not contribute to enzyme stability although it serves as a substrate and shows negative cooperativity.

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) from rabbit muscle has four chemically identical subunits (Harris and Perham, 1965) each of which binds NAD. The binding of NAD to the enzyme exhibits negative cooperativity (Conway and Koshland, 1968; DeVijlder and Slater, 1968), the apparent dissociation constants of the complex increasing as successive subunit sites are occupied.

Recently Bell and Dalziel (1975) have reported the first determination of all four apparent dissociation constants for NAD and for NADH. The high affinity of the first two sites required that the K_d values be estimated from changes in enzyme fluorescence, for sensitivity sufficient to permit the use of very low enzyme concentration. For both NAD and NADH, the K_d values increased by factors of 9, 40, and 9 for the three successive binding steps.

Schlessinger and Levitzki (1974) used ϵ-NAD (1-N^6-ethenoadenine dinucleotide), a fluorescent analogue which can substitute for NAD in the catalyzed reaction, as a spectroscopic probe for the coenzyme binding sites. They found binding constants for the third and fourth ϵ-NAD molecules to be similar to those for NAD. However, the dissociation constants of the first and second molecules of ϵ-NAD, though higher than those of NAD, were too low ($<10^{-6}$ M) to determine exactly.

The phenomenon of negative cooperativity in NAD binding to this enzyme has been recognized since the first report by Conway and Koshland (1968). In some cases it has been related to the phenomenon of half-of-sites reactivity toward acylating or alkylating reagents (Schlessinger and Levitzki, 1974; Levitzki, 1974; Stallcup and Koshland, 1973). Schlessinger and Levitzki (1974) have proposed that interaction of ligands at the adenine subsite of the coenzyme binding site produces structural changes that can be transmitted to neighboring subunits leading either to half-of-sites reactivity or to negative cooperativity. They have suggested that the nicotinamide subsite does not directly participate in such interactions and that ligands bound at this subsite do not directly affect neighboring subunits.

We have examined the binding of two analogues of NAD to the enzyme. Nicotinamide hypoxanthine dinucleotide is an active coenzyme for this enzyme (Eby and Kirtley, 1971). We find that it binds with negative cooperativity but, unlike NAD, its binding fails to stabilize the enzyme against thermal denaturation. Adenosine diphosphoribose, which lacks the nicotinamide ring of NAD, binds without negative (or positive) cooperativity but its binding stabilizes the enzyme. The results suggest that the nicotinamide ring at the coenzyme subsite is necessary for negative cooperativity in ligand binding but is not essential for half-of-sites reactivity with this enzyme.

Experimental Procedure

Materials. DL-Glyceraldehyde-3-phosphate diethylacetal barium salt, from Sigma, was deionized on Dowex 50W (hydrogen form) and the resulting solution heated at 100 °C for 3 min to obtain the free aldehyde. NAD, adenosine diphosphoribose, and nicotinamide hypoxanthine dinucleotide (Grade I, 96% pure) were also from Sigma. The only nucleotide impurity detected in the nicotinamide hypoxanthine dinucleotide was 2% inosine diphosphoribose as reported by Sigma. Dithiothreitol was from Calbiochem. Special enzyme grade ammonium sulfate was from Schwarz/Mann. All other chemicals (reagent grade) were from Fisher or Baker.

Purification of Glyceraldehyde-3-phosphate Dehydrogenase. New Zealand white rabbits (8–9-lb females) were

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anesthetized with ether and bled from the carotid arteries, and the back and hind leg muscles were taken as the source of enzyme. The dehydrogenase was extracted by the method of Cori et al. (1948) in the presence of 1 mM EDTA and 1 mM dithiothreitol. Three times recrystallized enzyme was purified by passing it through a Sephadex G-100 column according to Hill et al. (1975) using 0.05 M sodium pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol buffer, pH 8.3. Enzyme purified in this manner usually had a specific activity of 135–145 units/mg.

Enzyme Assay. Glyceraldehyde-3-phosphate dehydrogenase activity was measured according to a modification of the Velick method (1955) using a Gilford spectrophotometer Model 242. The assay mixture contained the following: 4.0 mM DL-glyceraldehyde 3-phosphate, 2.0 mM NAD, 10 mM sodium arsenate, 10 mM EDTA, and 0.05 M sodium pyrophosphate buffer in a total volume of 1.0 ml. The pH of the assay mixture was 8.0. Assays were initiated by the addition of enzyme and the increase in absorption at 340 nm was followed at room temperature to obtain the initial rate of NAD reduction. The specific activity was expressed as micromoles of NAD reduced per minute per milligram of protein.

Preparation of Apoenzyme. A solution of holoenzyme dissolved in 0.05 M sodium pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 8.3, was stirred with acid-washed activated charcoal (Norit) (3–4 mg of charcoal per mg of protein) for 10 min at 30 °C followed by centrifugation and suction filtration through a double layer of filter paper. This treatment produced apoenzyme with an A_{280}/A_{260} of 1.85–2.00 and with 70–80% of the activity of untreated enzyme. According to a study made by Bloch (1970), this absorbance ratio represents an average of 0.1 mol of NAD bound per mol of enzyme. The molecular weight of glyceraldehyde-3-phosphate dehydrogenase was taken to be 145 000 (Harrington and Karr, 1965). Protein concentration was determined using the 280-nm extinction coefficients of Murdock and Koeppe (1964), 0.815 and 1.06 $cm^2\ mg^{-1}$ for apo- and holoenzyme, respectively.

Ultrafiltration. Binding of nicotinamide hypoxanthine dinucleotide or adenosine diphosphoribose to charcoal-treated glyceraldehyde-3-phosphate dehydrogenase was determined using a 10-ml Amicon ultrafiltration cell with a Diaflo PM-10 membrane. Enzyme (0.5–5 mg/ml) was incubated in a 4-ml volume with appropriate concentrations of NAD analogue for 10 min, the total amount of added analogue being determined spectrophotometrically in a Zeiss PMQ II at 249 nm using the molar extinction coefficient of 14 500 (Siegel et al., 1959). Incubation was followed by filtration of the free analogue under a pressure of 15–20 psi of nitrogen. Two milliliters of filtrate was collected. The concentration of free analogue in the filtrate was measured at 249 nm and was taken to be equal to the concentration of free analogue in the ultrafiltration cell. The difference between the total amount of analogue added to the cell and the amount free at equilibrium after filtration was used to calculate the concentration of enzyme-bound analogue. Concentrations of free nicotinamide hypoxanthine dinucleotide in the filtrate were also determined by enzymatic reduction of the compound using the millimolar extinction coefficient of 6.22 at 340 nm. Concentrations determined in this manner were within 2–3% of those measured at 249 nm. All filtrations were carried out at ambient temperature and no detectable temperature changes occurred during the filtration. Control filtration using only solutions of nicotinamide hypoxanthine dinucleotide in 0.05 M pyrophosphate buffer showed that no significant amount of analogue was bound to the PM-10 membrane.

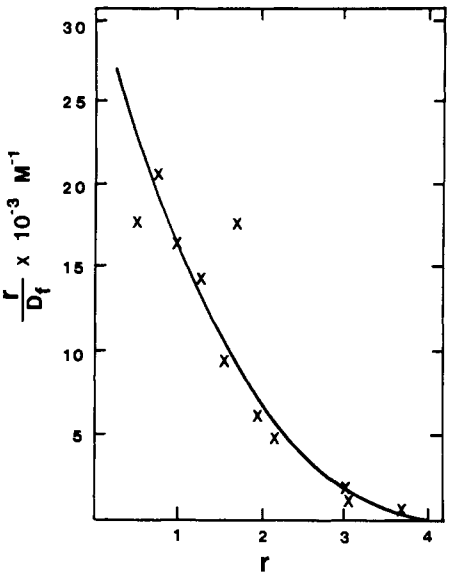

FIGURE 1: Binding of nicotinamide hypoxanthine dinucleotide to charcoal-treated glyceraldehyde-3-phosphate dehydrogenase at ambient temperature; A_{280}/A_{260} was 1.85–1.93. Enzyme and analogue were dissolved in 0.05 M sodium pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 8.3. The specific activity of the enzyme was 100–120 units/mg (after charcoal treatment). Enzyme (sites) concentration ranged from 13.4×10^{-6} to 155.2×10^{-6} M. The solid line is calculated assuming two types of binding sites using the values $n_1 = 1.9$, $n_2 = 2.0$, $K_{d_1} = 6.7 \times 10^{-5}$ M, $K_{d_2} = 1 \times 10^{-3}$ M. D_f is the concentration of free nicotinamide hypoxanthine dinucleotide, and r is the moles of bound analogue per mole of enzyme.

Substrate Activity. For K_m determinations, reaction rates were measured at 340 nm on the Gilford spectrophotometer. Final concentrations in the assay mixture were: 2.0 mM glyceraldehyde 3-phosphate, 10 mM sodium arsenate, 10 mM EDTA, 1.0 mM dithiothreitol, 0.05 M sodium pyrophosphate, pH 8.0. Analogue concentrations were varied. K_m values were obtained from Lineweaver-Burk plots of the experimental data.

Scatchard Plots. Biphasic Scatchard plots indicative of at least two pairs of binding sites were obtained for moles of analogue bound per mole of enzyme. The dissociation constants for the high and low affinity sites were determined by successive approximation (Weder et al., 1974).

Racker Band Titration. On binding of NAD to the enzyme an absorption band appears at 360 nm, the Racker band (Racker and Krimsky, 1952; DeVijlder and Slater, 1968). To a dialyzed solution of charcoal-treated glyceraldehyde-3-phosphate dehydrogenase were added increments of nicotinamide hypoxanthine dinucleotide. Absorption at 360 nm was measured as a function of added analogue using a Zeiss PMQ II spectrophotometer. Moles of analogue bound per mole of enzyme was calculated using the dissociation constants from the complex as obtained from the Scatchard plot.

Results

Ultrafiltration Binding Studies. The binding of nicotinamide hypoxanthine dinucleotide to glyceraldehyde-3-phosphate dehydrogenase was measured by ultrafiltration at several concentrations of enzyme and coenzyme analogue (Figure 1). A curve of this type is consistent either with nonidentical binding sites, or with negative cooperativity in binding to identical binding sites. There is general agreement that the latter explanation is correct for this enzyme. Because the charcoal-treated enzyme used in the binding studies has a

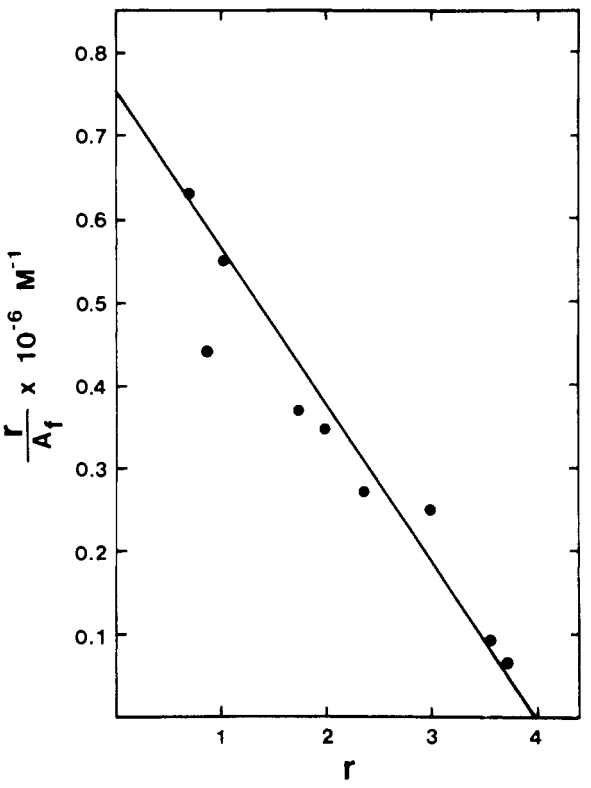

FIGURE 2: Scatchard plot for the binding of adenosine diphosphoribose to glyceraldehyde-3-phosphate dehydrogenase at 20–23 °C. Enzyme (sites) concentration ranged from 16.4×10^{-6} to 35.1×10^{-6} M. For conditions see Figure 1. A_f is the concentration of free adenosine diphosphoribose. From the slope the K_d is 5×10^{-6} M.

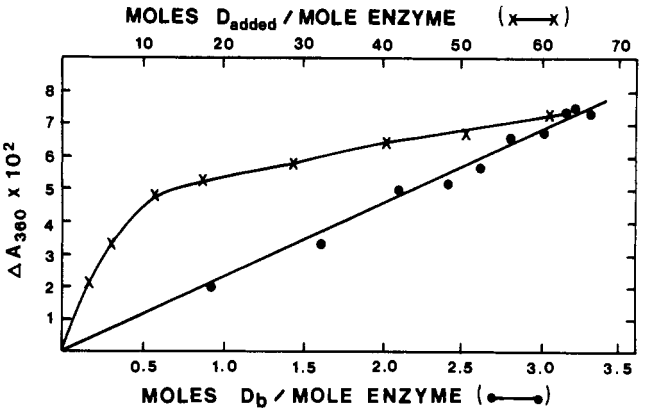

FIGURE 3: Titration of charcoal-treated glyceraldehyde-3-phosphate dehydrogenase with nicotinamide hypoxanthine dinucleotide. Charcoal-treated enzyme (30.0×10^{-6} M), A_{280}/A_{260} = 1.9, was titrated with the NAD analogue in 0.05 M pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 8.3 at 23 °C. The analogue was added in 0.01-ml increments in a 1-ml cuvette and readings were made at 360 nm. The upper curve gives the observed absorption after each addition (top axis). The amount of nicotinamide hypoxanthine dinucleotide bound to the enzyme after each addition was calculated using the constants from Figure 1. This is shown in the lower curve (bottom axis).

TABLE I: K_m Values for Nicotinamide Hypoxanthine Dinucleotide with Increasing Percent Saturation of 2.0×10^{-8} M Glyceraldehyde-3-phosphate Dehydrogenase.

Nicotinamide Hypoxanthine Dinucleotide Concn ($M \times 10^4$)	Initial Velocity (% of V_{max})	$K_{m_{app}}$ ($M \times 10^4$)
0.7–4.4	1–8	5.0
9.0–18.7	15–21	7.4
43.5–300.0	40–73	36.4

A_{280}/A_{260} ratio of about 1.9, indicating an average of 0.1 mol of NAD bound per mol of enzyme, it was assumed that there remain 1.9 high affinity sites and 2.0 low affinity sites per tetramer of the enzyme. The curve shown by the solid line was calculated using a dissociation constant of 6.7×10^{-5} M for the high affinity sites and 10^{-3} M for the low affinity sites.

In contrast, adenosine diphosphoribose gives a linear Scatchard plot throughout the saturation range (Figure 2). The four binding sites are indistinguishable in their affinity for this analogue. This result suggests that negative cooperativity in binding of coenzyme analogues requires the presence of the nicotinamide ring in the coenzyme structure.

Bell and Dalziel (1975) have found that binding of adenosine diphosphoribose results in quenching of fluorescence of the enzyme. Assuming that the quenching coefficient for each subunit is the same, they calculated apparent dissociation constants of 0.035 and 0.262×10^{-6} M for binding at the first two sites. Our direct binding studies show no evidence of negative cooperativity and give a calculated K_d of 5×10^{-6} M. The experimental conditions differ in the buffer used and in the pH (0.1 M triethanolamine, pH 7.6, vs. 0.05 M sodium pyrophosphate, pH 8.3). It is not yet possible to account for the difference in these results.

Substrate Activity of Nicotinamide Hypoxanthine Dinucleotide. In order to compare the binding results with the kinetic data, the K_m values for nicotinamide hypoxanthine dinucleotide were determined over a wide range of analogue concentrations at ambient temperature (Table I). In these experiments we determined the apparent K_m values at the high and low affinity sites. This was done using the double-reciprocal plots (1/velocity vs. 1/coenzyme concentration) using the experimental velocities in the ranges of 0–25% of V_{max}, 25–50% of V_{max} etc. The extrapolated intercept on the 1/coenzyme axis was used as the measure of -1/apparent K_m for the coenzyme in each velocity range. If there were no positive or negative cooperativity these apparent K_m values would be constant. As seen in Table I there is an obvious shift in overall apparent K_m with increasing coenzyme concentration consistent with negative cooperativity in coenzyme binding. Thus the negative cooperativity found in the binding studies with the analogue is also reflected in the initial velocity of the reaction of this analogue with glyceraldehyde-3-phosphate dehydrogenase.

Inhibition by Adenosine Diphosphoribose. The inhibition by adenosine diphosphoribose previously reported (Eby and Kirtley, 1971) has been confirmed and the inhibition curve shows no cooperativity in inhibitor binding either at low or at high concentrations of NAD.

Relation of Racker Band to Coenzyme Analogue Binding. Boers et al. (1971) have shown that the Racker band absorption produced by binding of NAD to glyceraldehyde-3-phosphate dehydrogenase is directly proportional to the occupancy of each site by the coenzyme. Binding of nicotinamide hypoxanthine dinucleotide to the enzyme also produces an increase in absorption at 360 nm due to Racker band formation (Figure 3, upper curve). The amount of analogue bound after each addition was calculated using the K_d values calculated above. As shown in Figure 3 (lower curve), the increase at 360 nm is linear with analogue binding up to 3.2 mol bound per mol of

enzyme. Saturation of the enzyme at higher concentrations of nicotinamide hypoxanthine dinucleotide was not possible due to instability of the enzyme so it is not certain whether occupancy of the fourth binding site by the analogue produces a proportional increase in Racker band absorption.

Discussion

Nicotinamide hypoxanthine dinucleotide was used as a structural analogue of NAD to study the coenzyme binding to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase since it binds less tightly than does NAD. It is apparent from the binding experiments (Figure 1) that the four subunits do not react independently of one another with the analogue. The Scatchard plot shows at least two pairs of sites, one pair having an affinity for the analogue 15 times that of the second pair. The data can be fitted using K_d values of 6.7×10^{-5} and 10^{-3} M for the two pairs of sites.

Neither the binding studies nor the kinetic data gave any evidence of difference in affinities of the first and second sites. The K_m values for nicotinamide hypoxanthine dinucleotide determined at initial velocities from 1.2 to 21.0% of the maximum were 5.0×10^{-4} and 7.4×10^{-4} M; whereas the K_m determined at velocities up to 72.5% of the maximum was 3.6×10^{-3} M. The K_m results show a decrease in affinity of the enzyme for nicotinamide hypoxanthine dinucleotide in going from the first to the second pair of sites and confirm negative cooperativity in binding of this ligand, as is the case with NAD.

All NAD analogues which are active coenzymes produce the Racker band absorption at 360 nm when bound to the enzyme (Eby and Kirtley, 1971). The spectrophotometric titration of apoglyceraldehyde-3-phosphate dehydrogenase with nicotinamide hypoxanthine dinucleotide results in an increase in absorbance at 360 nm up to 60 mol of ligand added per mol of enzyme (Figure 3). When these results are plotted on the basis of bound nicotinamide hypoxanthine dinucleotide, calculated from the K_d values, a linear increase in absorbance to 3.2 mol of ligand bound per mol of enzyme is seen (Figure 3, lower curve). This linear response with binding has also been observed with NAD binding (Boers et al., 1971). It suggests that the absorption increase is due to occupancy of each nicotinamide subsite and is not directly affected by changes transmitted from other subunits.

Levitzki (1974) and Schlessinger and Levitzki (1974) have linked the phenomena of half-of-sites reactivity of alkylating or acylating reagents and negative cooperativity in reversible ligand binding as effects that result from subunit conformational changes transmitted to neighboring subunits. They have proposed that those reagents which exhibit half-of-sites reactivity when covalently attached to the enzyme bind at the adenine subsite of the coenzyme binding site. They further suggested that all conformational changes transmitted between subunits are due to interaction at the adenine subsite and concluded that reagents occupying the nicotinamide subsite may result in local conformational changes but these are not transmitted to neighboring subunits. We suggest that, while these conclusions may be correct for the phenomenon of half-of-sites reactivity produced by covalently bound reagents, they do not necessarily apply to negative cooperativity in reversible ligand binding. Adenosine diphosphoribose shows no cooperative interactions with the enzyme either in binding studies (Figure 2) or as an inhibitor. Assuming that the adenine ring occupies the adenine subsite, we conclude that this is not sufficient to produce negative cooperativity under these conditions.

While the intact adenine ring is not required for negative cooperativity (as in nicotinamide hypoxanthine dinucleotide or ϵ-NAD) and is not sufficient to produce negative cooperativity (as in adenosine diphosphoribose), it plays an important role in the conformational stability of the enzyme. The thermal stability that results from occupancy of the adenine subsite by adenine in an NAD analogue may be due either to alteration of the subunit conformation or to stabilization of the preexisting conformation of the apoenzyme. While we cannot distinguish between these, it is still clear that the stabilization effect is not necessary for substrate activity of a bound coenzyme or for negative cooperativity in its binding. Nicotinamide hypoxanthine dinucleotide is a good example of an active coenzyme which fails to stabilize but which binds with negative cooperativity.

We suggest the following functions for the adenine subsite and nicotinamide subsite. Occupancy of the adenine subsite is necessary but is not always sufficient to produce negative cooperativity in ligand binding. Occupancy of the adenine subsite by adenine in NAD analogues leads to stabilization of the enzyme. Alkylating or acylating reagents which occupy the adenine subsite can result in half-of-sites reactivity (Levitzki, 1974). Binding at the nicotinamide subsite is necessary for substrate activity and for Racker band formation. Alkylating or acylating reagents which occupy this site do not lead to half-of-sites reactivity (Levitzki, 1974). In some cases occupancy of the nicotinamide subsite may be necessary for negative cooperativity in ligand binding.

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