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Mechanism of negative cooperativity in glyceraldehyde-3-phosphate dehydrogenase deduced from ligand competition experiments

(site–site interactions/NAD^+)

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ABSTRACT **It is shown that the modulation in the negative cooperativity of ligand binding by another, competing ligand that binds noncooperatively is accounted for exclusively by the ligand-induced sequential model. It is therefore suggested that whenever such a phenomenon is observed it argues strongly in favor of the sequential model. The advantages and limitations of this approach are evaluated. The binding of the coenzymes NAD^+ and nicotinamide-1-N^6-ethenoadenine dinucleotide to rabbit muscle apo-glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating; EC 1.2.1.12] exhibits strong negative cooperativity, whereas acetylpyridine adenine dinucleotide, ATP, and ADP-ribose bind noncooperatively to the NAD^+ sites. The strong negative cooperativity in coenzyme binding was found to be abolished in the presence of acetylpyridine adenine dinucleotide and strongly weakened by ATP, ADP, and AMP, but was not affected by addition of ADP-ribose. These findings demonstrate that the negative cooperativity in coenzyme binding to this enzyme results from sequential conformational changes and exclude the pre-existent asymmetry model as a possible explanation. These results also support the view that the structure of the pyridine moiety of the coenzyme analogs plays a role in orienting the adenine moiety at the adenine subsite, therefore affecting the cooperativity in the binding of the coenzyme analog which is mediated through the adenine subsites.**

Rabbit muscle glyceraldehyde-phosphate dehydrogenase (Gra-*P* dehydrogenase) [D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating); EC 1.2.1.12] exhibits negative cooperativity in the binding of the coenzyme NAD^+ (1, 2) and some of analogs (3–5). Similar findings were reported for the lobster muscle enzyme (6, 7), the sturgeon muscle enzyme (8), and the *Bacillus stearothermophilus* enzyme (9). Negatively cooperative binding can, in principle, result from one of two situations: pre-existent asymmetry (heterogeneity) with two or more classes of binding sites, or ligand-induced decrease in the affinity of the protein towards further ligand binding. The pre-existent asymmetry model has been used by Bernhard and his colleagues to explain the behavior of the rabbit muscle enzyme (10, 11) and of the sturgeon muscle enzyme (8, 12). The ligand-induced sequential model (13) has also been used to explain the behavior of the rabbit muscle enzyme (1–3, 5, 14). Keleti *et al.* (15) suggested that the negatively cooperative binding of NADH to rabbit muscle Gra-*P* dehydrogenase is due to the dissociation of the tetramer to dimers and monomers; the dissociated species bind the coenzyme more tightly than the nondissociated tetramer. Although theoretically possible, it seems that such a theory cannot account for the binding studies quoted above and those reported in this study. Under the experimental conditions used in the studies quoted above and presented here (a tetramer concentration above 10 μM), Gra-*P* dehydrogenase is exclusively in its tetrameric form, as was demonstrated by Hoagland and Teller (16).

X-ray crystallographic data did not clearly distinguish between the pre-existent asymmetry model and the sequential model. Rossmann and his group reported possible asymmetry in lobster muscle holoenzyme (17, 18), although preliminary experiments with the apoenzyme (18) imply that a conformational change probably occurs upon removal of the last NAD^+ molecule from the enzyme. Thus, the asymmetry observed in the holoenzyme may well be *induced* by NAD^+ binding. X-ray data on the enzyme from *B. stearothermophilus* did not reveal any asymmetry either in the holoenzyme or in the apoenzyme. Both forms were reported to possess an exact 2:2:2 tetrahedral symmetry, and conformational changes were observed upon removal of the bound NAD^+ (19, 20).

In the present study we describe a method (21), based on ligand competition experiments, for establishing the mechanism of the negative cooperativity of coenzyme binding to rabbit muscle Gra-*P* dehydrogenase.

EXPERIMENTAL PROCEDURES

Materials. Gra-*P* dehydrogenase was prepared from rabbit muscle as described (5). Apoenzyme was prepared by charcoal treatment (5). ATP, NAD^+, adenosine diphosphoribose (ADP-Rib), and acetylpyridine adenine dinucleotide (Ac$PyAD^+$) were obtained from Sigma. Ac$PyAD^+$ was further freed from NAD^+ contamination by passing 23 ml of a mixture containing 0.1 mM apoenzyme and 1.32 mM Ac$PyAD^+$ through a Sephadex G-25 column (5.2 × 50 cm) pre-equilibrated with 5 mM NH_4HCO_3 (pH 8.4).‡ Nicotinamide-1-N^6-ethenoadenine dinucleotide (ϵNAD^+) was prepared according to Barrio *et al.* (22) and further purified as described (3). Nicotinamide [U-^{14}C]adenine dinucleotide (302 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels) and [α-^{32}P]ATP (7.7 Ci/mmol) were obtained from Radiochemical Centre (Amersham, England).

Binding Measurements of Coenzyme Analogs. All binding and competition measurements were performed in 50 mM Hepes/10 mM EDTA, pH 7.5, at 25°C. All measurements used

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Abbreviations: KNF model, the Koshland–Neméthy–Filmer model; PEA model, the pre-existent asymmetry model; Gra-*P* dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); ϵNAD^+, nicotinamide-1-N^6-ethenoadenine dinucleotide; Ac$PyAD^+$, acetylpyridine adenine dinucleotide; ADP-Rib, adenosine diphosphoribose.

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‡ Because of the much higher affinity of NAD^+ for the enzyme, all the NAD^+ is removed with the enzyme peak and an Ac$PyAD^+$ peak with no NAD^+ contamination follows.

tetramer concentrations of 8 μM or higher in order to ensure no significant dissociation into dimers, which occurs only at *lower* enzyme concentrations (16).

AcPyAD$^+$ binding was followed by the difference spectrum band formed upon its binding to the enzyme at 350 nm (23), as described (5). As for other NAD$^+$ analogs (3, 5, 24), the intensity of this band was proportional to AcPyAD$^+$ occupancy, with ϵ_{350} = 1430 M^{-1} cm^{-1} for bound AcPyAD$^+$.

ϵNAD$^+$ binding was measured by the enhancement in ϵNAD$^+$ fluorescence upon binding to the enzyme, by using a Hitachi–Perkin Elmer MPF-4 spectrofluorimeter at 25°C, with a 1.0-mm optical path cuvette positioned at 45° to the exciting and emitted light. The fluorescence was excited at 325 nm, and the emission was recorded at 389 nm. When the ϵNAD$^+$ concentration exceeded 0.12 mM, a correction for the decrease in fluorescence due to the absorption of the exciting light was required.[§] This correction did not exceed 5% in all cases.

The binding of ATP and NAD$^+$ was measured by the dialysis rate method of Collowick and Womack (25) with radioactive ligands. The titrating solution contained protein and radioactive ligand at concentrations identical to those in the titrated solution to prevent dilution during the titration. The dialysis cell used was 1 × 1 × 1 cm in the upper chamber and 0.1 ml volume in the lower chamber. The separating membrane was a Visking cellulose tubing. The flow rate was 2 ml/min, and 10 samples (1 ml each) were collected subsequent to each addition of ligand to the upper chamber (steady state was obtained after 5 ml).

Analysis of Binding Curves. Binding data were analyzed by computer with a nonlinear regression curve-fitting library program (26). The analysis was usually performed with the general Adair equation for ligand binding to a tetramer (27):

$$\frac{[X]_B}{[E]_t} = \frac{\frac{[X]}{K_1} + \frac{2[X]^2}{K_1K_2} + \frac{3[X]^3}{K_1K_2K_3} + \frac{4[X]^4}{K_1K_2K_3K_4}}{1 + \frac{[X]}{K_1} + \frac{[X]^2}{K_1K_2} + \frac{[X]^3}{K_1K_2K_3} + \frac{[X]^4}{K_1K_2K_3K_4}}, \quad [1]$$

in which $[E]_t$ is the total tetramer concentration, $[X]_B$ and $[X]$ are the concentrations of bound and free ligand, respectively, and K_1 through K_4 are the thermodynamic dissociation constants for the binding of the first through the fourth ligand molecules to the tetramer.

RESULTS

Theory. In the present study we used a new approach (21) to establish the mechanism of the negative cooperativity in rabbit muscle Gra-*P* dehydrogenase. The Koshland–Némethy–Filmer (KNF) and the pre-existent asymmetry (PEA) models predict different effects of noncooperative competing ligands on the mode of binding of a negatively cooperative ligand (21). The same method can also be used to distinguish between the model of Monod *et al.* (28) and the KNF model for positive cooperativity (21).

The Hill coefficient is a widely used parameter for cooperativity, and we have chosen to explore the behavior of that coefficient as predicted by the different allosteric models. For a dimer or a tetramer possessing two classes of noninteracting sites (the PEA model), the Hill coefficient at 50% saturation by a ligand X is given by (29, 30):

$$n_H(X) = \frac{4}{2 + (K'_X/K''_X)^{1/2} + (K''_X/K'_X)^{1/2}}, \quad [2]$$

in which K'_X and K''_X are the intrinsic affinity constants of the ligand X to the two classes of sites. When a competing ligand Z is present in a concentration much higher than the concentration of the binding sites (so that the free Z concentration is essentially constant during the titration with X), the Hill coefficient for X at 50% saturation with X, $n_H(X,Z)$, becomes (21):

$$n_H(X,Z) = 4 \div \left(2 + \frac{K'_X + K''_X + (K'_XK''_Z + K'_ZK''_X)[Z]}{\sqrt{K'_XK''_X\{1 + (K'_Z + K''_Z)[Z] + K'_ZK''_Z[Z]^2\}^{1/2}}}\right), \quad [3]$$

in which K'_Z and K''_Z are the intrinsic affinity constants of Z to the two classes of sites. If the ligand Z binds noncooperatively, $K'_Z = K''_Z$ and Eq. 3 reduces to Eq. 2; i.e., when the competing ligand Z binds noncooperatively, $n_H(X,Z) = n_H(X)$ and the cooperativity in X binding remains unchanged according to the PEA model (21).

In contrast, the KNF model allows for a change in the cooperativity of X binding when the competing ligand binds noncooperatively. In the simplest case of the KNF model, in which the conformational change induced upon X binding is limited to the ligand-binding subunit, one obtains for a dimer (29, 30):

$$n_H(X) = \frac{2}{1 + (K^2_{AB}/K_{BB})^{1/2}}, \quad [4]$$

in which K_{AB} and K_{BB} are the subunit interaction constants between an X-liganded conformation (B) and an unliganded conformation (A) and between two X-liganded conformations, respectively (13). In the presence of a high concentration of a competing ligand Z, one obtains (21):

$$n_H(X,Z) = 2 \div \left(1 + \left[\frac{K^2_{AB}(1 + K_{BC}K_{Z_C}K_{t_{AC}}[Z]/K_{AB})^2}{K_{BB}\{1 + 2K_{AC}K_{Z_C}K_{t_{AC}}[Z] + K_{CC}(K_{Z_C}K_{t_{AC}})^2[Z]^2\}}\right]^{1/2}\right) \quad [5]$$

in which K_{AC} and K_{BC} are the subunit interaction constants between a Z-liganded subunit (conformation C) and an unliganded subunit (conformation A) and between the Z-liganded subunit and an X-liganded subunit (conformation B), respectively. $K_{t_{AC}}$ describes the free energy of the transformation of a subunit from conformation A to conformation C (13), and K_{Z_C} is the intrinsic affinity constant of Z to a subunit in the C conformation. In the KNF model, a ligand Z binds noncooperatively only if $K^2_{AC}/K_{CC} = 1$ (13). However, K_{BC}/K_{AB} in Eq. 5 can assume any value. Thus, according to the KNF model, Eq. 5 does not reduce to Eq. 4 even if Z binds noncooperatively, and $n_H(X,Z) \neq n_H(X)$. Only in the special case where $K_{BC}/K_{AB} = K_{AC}$ will $n_H(X,Z)$ equal $n_H(X)$. Eq. 5 also applies to a tetramer that behaves as a dimer of dimers (21). A similar behavior is found for the general tetramer case, for which more involved algebraic manipulations must be applied (21). To summarize this point, the KNF model can account for a change in the cooperativity of X binding in the presence of a noncooperative competing ligand whereas the PEA model cannot. A full theoretical treatment of the tetramer case is available and can be extended to higher oligomers (21).

Binding of Ligands to Gra-*P* Dehydrogenase. The binding of NAD$^+$ and of ϵNAD$^+$ to Gra-*P* dehydrogenase exhibits the

[§] In the absence of enzyme, the fluorescence intensity of free ϵNAD$^+$ plotted against its concentration is linear up to 0.12 mM. At higher ϵNAD$^+$ concentrations, the ratio between the extrapolated straight line and the actual ϵNAD$^+$ fluorescence intensity yields a correction factor for that specific ϵNAD$^+$ concentration. The fluorescence intensity observed for this ϵNAD$^+$ concentration in the presence of the enzyme is then multiplied by that factor to give the corrected fluorescence intensity.

phenomenon of negative cooperativity. The binding of AcPyAD$^+$, ATP, and ADP-Rib to the coenzyme binding sites is noncooperative. Thus it is possible to explore the effect of the noncooperative binders on the binding of the negatively cooperative coenzyme molecules.

Binding of ϵNAD$^+$. Under conditions where all of the ϵNAD$^+$ is bound to the enzyme (5.5 μM ϵNAD$^+$, 50 μM apoenzyme), a 6-fold fluorescence enhancement factor was obtained at the emission wavelength of 389 nm. The fluorescence enhancement (ΔF) was proportional to ϵNAD$^+$ binding as reported (3). The maximal fluorescence enhancement ΔF_∞ was obtained when the enzyme was fully saturated with ϵNAD$^+$. The ratio $\Delta F/\Delta F_\infty$ gives the fraction of sites occupied by ϵNAD$^+$; i.e., $[\epsilon NAD^+]_B/4[E]_t$. The binding of ϵNAD$^+$ to the enzyme exhibited strong negative cooperativity (Fig. 1 and Table 1). NAD$^+$ binding also showed strong negative cooperativity (Fig. 1), as reported earlier (1, 2).

On the other hand, the binding of AcPyAD$^+$ and of ATP was noncooperative (Fig. 1 and Table 2). For ATP, the noncooperative binding to four sites shown in Fig. 1 was measured in the presence of 5 mM P_i in order to block ATP binding to the eight P_i-binding sites on the enzyme (18). In the presence of 5 mM NAD$^+$, which blocks ATP binding to the NAD$^+$ sites, the four tighter ATP-binding sites disappeared and eight low-affinity sites, exhibiting an intrinsic dissociation constant of (7.0 $\pm$ 1) $\times$ 10^{-4} M, were observed (data not shown). This dissociation constant agrees with the inhibition constant of ATP with respect to P_i in the enzyme-catalyzed reaction, which is 8 $\times$ 10^{-4} M at pH 7.4 (33).

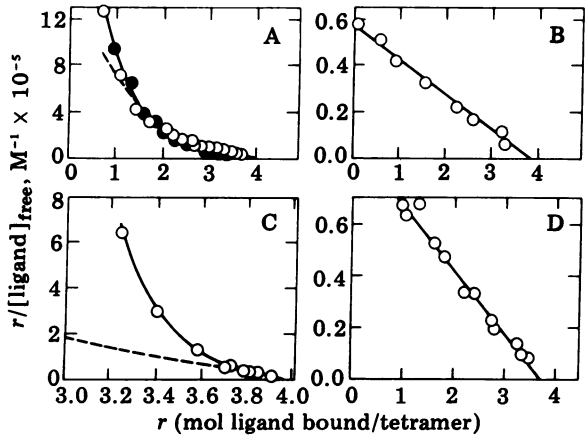

FIG. 1. Binding of coenzyme analogs to Gra-*P* dehydrogenase. Data are presented in the form of Scatchard plots (31). (*A*) ϵNAD$^+$ binding. The enzyme concentration was 8.4 μM. ● and ○, Two separate experiments; —, best fit to Eq. 1; ---, best fit to the two-classes-of-sites (PEA) model $[X]_B/[E]_t = 2[X]/(K' + [X]) + 2[X]/(K'' + [X])$, in which K' and K'' are the intrinsic dissociation constants of the ligand X to the two classes of sites, respectively. The data are not sufficient to clearly eliminate the latter model, which is the simplest case of the PEA model for a tetramer, although it has only two parameters (compared to four parameters in Eq. 1). The correlation coefficients are 0.9997 for Eq. 1 and 0.9995 for the PEA model. (*B*) ATP binding. The enzyme concentration was 51 μM; the [α-^{32}P]ATP concentration was 2.6 μM (7.7 Ci/mmol). All solutions contained, in addition, 5 mM P_i. Nonspecific binding of ATP to the dialysis membrane in the flow–dialysis method was measured by titrating with ATP in the absence of enzyme. The nonspecific binding was subtracted in all cases. (*C*) NAD$^+$ binding. The enzyme concentration was 10 μM; the [^{14}C]NAD$^+$ concentration was 7.4 μM (302 Ci/mol). No nonspecific binding of NAD$^+$ to the dialysis membrane was observed. ---, Limiting slope at infinite saturation which can be used to calculate the affinity of NAD$^+$ to the fourth binding site. (*D*) AcPyAD$^+$ binding. The enzyme concentration was 27.5 μM. Bound AcPyAD$^+$ was computed by using ϵ_{350} = 1430 M^{-1} cm^{-1}.

Table 1. Intrinsic dissociation constants* for ϵNAD$^+$ binding to the enzyme

Intrinsic dissociation constant	Value, M $\times$ 10^5 This study†	From ref. 3	From ref. 32
K'_1	0.15 $\pm$ 0.03	ND	ND
K'_2	0.63 $\pm$ 0.09	ND	ND
K'_3	1.30 $\pm$ 0.20	1.7	3.5
K'_4	1.50 $\pm$ 0.20	1.7	3.5

ND, not determined. K'_i are the intrinsic dissociation constants for the binding of the ith ϵNAD$^+$ molecule. The constants in this study were obtained by curve-fitting by using Eq. 1. The constants from ref. 3 were obtained by reanalyzing the data from ref. 3 by using Eq. 1. The constants from ref. 32 were obtained by using Gra-*P* dehydrogenase with two NAD$^+$ molecules bound; clearly the presence of bound NAD$^+$ might affect the affinity of the vacant sites towards ϵNAD$^+$.

* The intrinsic dissociation constants are related to the thermodynamic dissociation constants by the formula (29): $K'_i = [(n - i + 1)/i]\, K_i$, in which K_i is the thermodynamic dissociation constant for the binding of the ith ligand molecule to EX_{i-1}, and K'_i is the corresponding intrinsic dissociation constant. n is the number of binding sites on the enzyme.

† SDs are shown.

Effect of Noncooperative Ligands on Coenzyme Binding. Competition experiments were carried out primarily with ϵNAD$^+$ because it was possible to characterize the full binding curve of this analog to the enzyme (unlike NAD$^+$) and to obtain an accurate n_H value characterizing its binding. The competing noncooperative NAD$^+$ analogs were always introduced in a large molar excess over the binding sites and over their dissociation constants so that the concentration of free competing ligands did not change upon titration with the primary ligand. The large excess over the competing ligand's dissociation constant ensures that the binding sites are initially saturated with the competing ligand and, thus, that the maximal possible effect of the competing ligand on the cooperativity of the primary ligand can be obtained.

The results of the competition experiments with ϵNAD$^+$ are summarized in Fig. 2 and Table 3. In the presence of saturating AcPyAD$^+$ concentrations, the negative cooperativity in the binding of ϵNAD$^+$ to the enzyme was completely abolished (Fig. 2 and Table 3). ATP concentrations that nearly saturate the NAD$^+$ binding sites severely weakened the negative cooperativity in ϵNAD$^+$ binding (Fig. 2 and Table 3). The effect of ATP on the cooperativity of ϵNAD$^+$ binding is entirely due to its competition on the NAD$^+$-binding sites and not to noncompetitive interaction of ATP with the P_i-binding sites. This was proven by the findings that ATP affected the cooperativity

Table 2. Intrinsic dissociation constants for the binding of noncooperative NAD$^+$ analogs to Gra-*P* dehydrogenase

Ligand	Intrinsic dissociation constant, M $\times$ 10^5
ATP	6.7 $\pm$ 0.7
AcPyAD$^+$	4.0 $\pm$ 0.4
ADP-Rib	4.5 $\pm$ 0.5

The constants for ATP and AcPyAD$^+$ were derived from the experiments shown in Fig. 1. The effect of ADP-Rib on the binding of ϵNAD$^+$ to the enzyme was purely competitive (Fig. 2; see text), and thus the dissociation constant for ADP-Rib could be derived from its competition with ϵNAD$^+$, described by: $[\epsilon NAD^+]^{obs}_{0.5} = [\epsilon NAD^+]_{0.5}\,(1 + ([\text{ADP-Rib}]/K'))$, in which $[\epsilon NAD^+]_{0.5}$ and $[\epsilon NAD^+]^{obs}_{0.5}$ are the free ϵNAD$^+$ concentrations yielding 50% saturation in the absence and the presence of ADP-Rib, respectively. K' is the intrinsic dissociation constant for ADP-Rib binding.

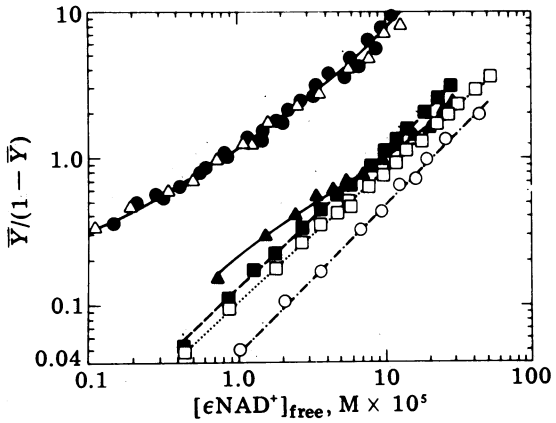

FIG. 2. Effect of noncooperative competing ligands on ϵNAD^+ binding. Data are presented in the form of Hill plots (34). $\overline{Y} = [\epsilon NAD^+]_{bound}/4[E]_{total}$. ●—●, binding of ϵNAD^+ alone; △—△, binding of ϵNAD^+ in the presence of 5 mM P_i; □·····□, binding of ϵNAD^+ in the presence of 0.6 mM ATP; ■- - ■, binding of ϵNAD^+ in the presence of 0.63 mM ATP and 5 mM P_i; ○···○, binding of ϵNAD^+ in the presence of 0.41 mM $AcPyAD^+$; ▲—▲, binding of ϵNAD^+ in the presence of 0.53 mM ADP-Rib. The enzyme concentration in all experiments was between 8 and 9 μM.

of ϵNAD^+ binding to the same extent in the presence and in the absence of P_i and that P_i itself had no effect at all on the cooperativity of ϵNAD^+ binding (Fig. 2). The effects of ATP and $AcPyAD^+$ on the cooperativity of ϵNAD^+ binding were also not due to any influence these ligands might have had on the association–dissociation equilibria of the tetramer; ultracentrifugation experiments (sedimentation velocity) under conditions identical with those used in the competition experiments revealed no effect of ATP and $AcPyAD^+$ on the state of aggregation of the tetramer (data not shown). This agrees with the reports that at 20°C, ATP does not cause dissociation of the tetrameric enzyme (35).

Unlike the marked effects of $AcPyAD^+$ and ATP, ADP-Rib had no effect on the shape of the ϵNAD^+ binding curve but only shifted it to higher ϵNAD^+ concentrations. Such a phenomenon can be encountered only if the competing ligand binds noncooperatively and, in addition, if its binding is not affecting (and not affected by) the binding of ϵNAD^+ to neighboring subunits (21). These results confirm previous findings that ADP-Rib binds noncooperatively to Gra-*P* dehydrogenase (36).

The binding of the first three NAD^+ molecules to the enzyme is too tight to permit accurate determination of n_H for the binding of NAD^+ alone. Therefore, we restricted our measurements to the comparison between n_H values for NAD^+ binding in the presence of different noncooperative analogs. The results are presented in Fig. 3 and Table 3. The effects of

Table 3. n_H values for binding of ϵNAD^+ and NAD^+ to the enzyme in the presence of noncooperative competitive ligands

Competing ligand	$n_H(\epsilon NAD^+)$	$n_H(NAD^+)$
None	0.64 ± 0.03	ND*
ADP-Rib	0.65 ± 0.04	0.58 ± 0.04
$AcPyAD^+$	1.00 ± 0.04	ND
ATP	0.89 ± 0.02	0.88 ± 0.04

ND, not determined. The Hill coefficients were obtained from the slope of the Hill plots at midpoint (Figs. 2 and 3). Each experiment was repeated at least three times, and the standard deviation was determined by using SD = $\sqrt{\sum(\bar{n}_H - n_H)^2/N}$.

* n_H for the binding of NAD^+ alone could not be determined because of the tight binding of the first three NAD^+ molecules to the enzyme.

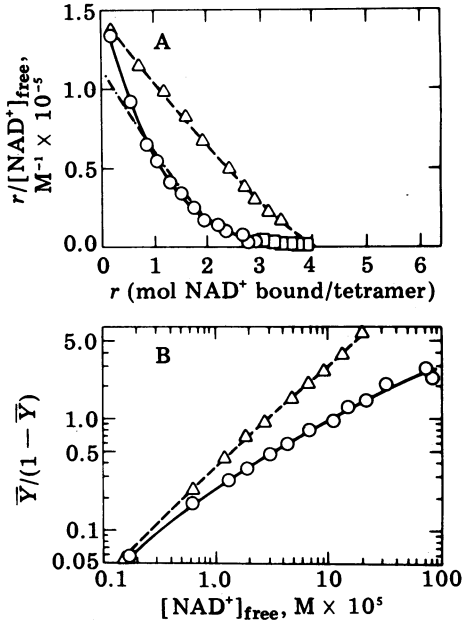

FIG. 3. NAD^+ binding in the presence of ADP-Rib and ATP. The enzyme concentration was 27 μM; the $[^{14}C]NAD^+$ concentration was 7.4 μM (302 Ci/mol). ○—○ and □—□, Titration in the presence of 10.45 mM ADP-Rib, best fit according to Eq. 1; ○···○, same titration in the presence of ADP-Rib, best fit according to the PEA model (for details, see legend to Fig. 1*A*); △- - △, titration in the presence of 11.85 mM ATP, best fit according to Eq. 1. As in the case of ϵNAD^+ binding, NAD^+ binding (*A*) can be fitted well also to the PEA model. (*A*) Scatchard plots; (*B*) Hill plot. $\overline{Y} = [NAD^+]_{bound}/4[E]_{total}$.

ATP and ADP-Rib on NAD^+ binding were strikingly different from each other, and the n_H values obtained in both cases correlated closely with the corresponding n_H values for ϵNAD^+ when the latter was used as the primary ligand (Table 3).

Assuming that ADP-Rib binding does not affect the cooperativity of NAD^+, similar to its lack of effect on the cooperativity of ϵNAD^+ binding, all four intrinsic NAD^+ dissociation constants were calculated from the binding curve of NAD^+ in the presence of 10.46 mM ADP-Rib (Table 4).

Table 4. Intrinsic dissociation constants for NAD^+ binding to Gra-*P* dehydrogenase

Intrinsic dissociation constants	Values, M × 10^6		
	This study	From ref. 1	From ref. 2
K'_1	0.11 ± 0.03	<4.0 × 10^{-5}	<0.1
K'_2	0.35 ± 0.09	<1.5 × 10^{-3}	<0.1
K'_3	0.59 ± 0.13	0.3	2.7
K'_4	5.40 ± 1.00	5.0	8.2

The intrinsic dissociation constants K'_1 through K'_4 were obtained from the respective best-fit dissociation constants for the binding of NAD^+ in the presence of 10.47 mM ADP-Rib (Fig. 3). This calculation was performed by using the formula: $K^{obs}_{X_i} = K'_{X_i}(1 + ([\text{ADP-Rib}]/K'_{\text{ADP-Rib}}))$, in which $K^{obs}_{X_i}$ and K'_{X_i} are the intrinsic dissociation constants for the binding of the *i*th molecule of NAD^+ in the presence and absence of ADP-Rib, respectively. This formula is obtained on the assumption that all four intrinsic dissociation constants for NAD^+ binding are influenced by ADP-Rib competition in an identical manner, as is the case for ϵNAD^+. The values reported earlier for K'_1 and K'_2 (1, 2) are rough estimates only, because of the high tetramer concentration (20 μM) used and the NAD^+ tight binding. Studies with very low tetramer concentration are cumbersome because the apo-enzyme tetramer dissociates to dimers and tends to denature (35).

DISCUSSION

Sequential Nature of Coenzyme Binding. A significant decrease in the extent of negative cooperativity of ϵNAD^+ to Gra-*P* dehydrogenase binding is observed in the presence of saturating concentrations of the noncooperative analogs Ac-PyAD$^+$ and ATP (Fig. 2 and Table 3). These findings clearly eliminate a PEA model without ligand-induced changes and establish that the mechanism of negative cooperativity in coenzyme binding to the rabbit muscle enzyme involves sequential conformational changes (13, 29, 37, 38).

Interestingly, the noncooperative NAD$^+$ analog ADP-Rib has no effect on the cooperativity of ϵNAD^+ binding (Fig. 2 and Table 3). This indicates the need for using more than one noncooperative competing ligand in the study of a cooperative mechanism; different competing ligands may possess (or lack) different effects on the cooperativity of the enzyme towards the primary ligand, depending on their specific interactions with the enzyme.

The Hill coefficients for the binding of ϵNAD^+ and NAD$^+$ to Gra-*P* dehydrogenase in the presence of saturating concentrations of ADP-Rib or ATP are almost identical to each other (Fig. 3 and Table 3). It seems, therefore, that the conclusions drawn from the competition experiments with ϵNAD^+ also hold for the natural coenzyme, NAD$^+$.

Mode of Coenzyme Binding. Previous evidence indicated participation of the adenine subsites in the transmission of conformational changes in Gra-*P* dehydrogenase (4, 14). No conformational changes were detected in the nicotinamide subsites (5). Nevertheless, modifications in the pyridine moiety significantly affect the cooperativity of coenzyme binding, probably by changing the mode of interaction of the enzyme with the adenine moiety. Indeed, AcPyAD$^+$ binds noncooperatively to the enzyme (ref. 23 and Fig. 1) although it differs from NAD$^+$ only in the pyridine moiety.

Unlike AcPyAD$^+$, ADP-Rib does not affect the cooperativity of ϵNAD^+ binding, probably because it lacks a pyridine moiety altogether. ATP affects the mode of ϵNAD^+ binding although it also lacks a pyridine moiety. However, several findings indicate that ATP binds to the enzyme in a mode very different from that of all other NAD$^+$ analogs, most likely due to the ionic character of ATP binding.

To summarize, coenzyme binding to Gra-*P* dehydrogenase includes a conformational change which is transmitted to neighboring subunits via the adenine subsites. The binding of the pyridine moiety to the nicotinamide subsite affects the orientation of the adenine moiety at the adenine subsite, thus determining the nature of the conformational transitions at the neighboring subunits, and therefore determines the mode of binding of the ligand as a whole.

Rigorous Nature of the Method. The approach described in this study uses equilibrium binding experiments exclusively. Therefore, it is possible, on the basis of competition experiments alone, to determine the mode of binding of a ligand. In this communication we have used this approach to determine the mechanism for the negatively cooperative binding case; the same method can be adopted for distinguishing between the concerted model (28) and the KNF model in the case of positive cooperativity (21). This general approach can easily be adopted for analysis of ligand binding to receptors, many of which reveal the phenomenon of negative cooperativity.

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