Positive and Negative Cooperativity in Yeast Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: Binding studies of nicotinamide–adenine dinucleotide to yeast glyceraldehyde 3-phosphate dehydrogenase reveal significant deviations from the normal Michaelis–Menten behavior. The binding data show positively cooperative behavior but the overall nature of the curves suggest greater complexity. A break occurs on the Hill plot at approximately 50% saturation and the Scatchard plots do not extrapolate to four which would be expected on the basis of the number of subunits. The anomalies can be explained by a ligand-induced sequential model with subunit interactions which lead to a mixture of positive and negative cooperativity.

Assuming a homogeneous protein, binding constants in the relationship $K_1' < K_2' > K_3' > K_4'$ show an excellent fit to the experimental data. The simpler models do not fit as well.

D-Glyceraldehyde 3-phosphate dehydrogenase was first crystallized from yeast by Warburg and Christian (1939) and has been the subject of intensive research efforts since that time. Despite the accumulated knowledge about the yeast enzyme, there are many confusing anomalies in the literature. Crystalline glyceraldehyde 3-phosphate dehydrogenase from yeast was originally thought to have no bound NAD when prepared according to the methods of Warburg and Christian (1939) or Krebs *et al.* (1953). By adding NAD to the crystalline preparation with a OD_{280}:OD_{260} ratio of 1.5, both Velick (1953) and Stockell (1959) concluded that 2 moles of NAD was bound per mole of enzyme. By treating the crystalline enzyme with charcoal to remove bound NAD it has been shown to raise the OD_{280}:OD_{260} ratio to >2.0. By adding NAD to the charcoal-treated enzyme, both Kirschner *et al.* (1966) and Chance and Park (1967) concluded that 4 moles of NAD was bound per mole of enzyme.

The equality or inequality of the NAD binding sites is also a controversial issue. For example, Velick (1953) has shown that the K_{NAD} of the yeast enzyme is dependent upon the amount of bound NAD; the K_{NAD} being 1.7×10^{-5} mole/l. when 0.5 mole of NAD bound and a K_{NAD} of 1.9×10^{-4} mole/l. when 2.0 moles of NAD bound. This variation in affinity was not observed by Stockell (1959) who concluded that the two sites were independent and equal with a K_{NAD} of 4.5×10^{-4} mole/l. In the case of the charcoal-treated GPD,[1] Kirschner *et al.* (1966) concluded that the four NAD binding sites were independent and equal at 25° by spectrophotometric titration. By use of fast-flow techniques, Chance and Park (1967) were able to measure the formation of the enzyme–NAD complex and the formation of reduced NAD in the overall oxidation of GPD simultaneously. The NAD binding sites were found to be unequal as indicated by biphasic kinetics for compound formation, differential reactivity to *p*-mercuribenzoate or acetyl phosphate, and selective reactivity in the overall oxidation process. Since GPD has been shown to be a tetramer with apparently identical monomers (Harris and Perham, 1965), the differences in the NAD binding sites appear not to be due to the amino acid sequence of the monomers.

The controversy over the identity or nonidentity of the NAD binding sites of the yeast GPD coupled with the explanation of the mechanism of the rabbit muscle GPD in terms of negative cooperativity (Conway and Koshland, 1968) prompted an examination of the yeast enzyme. The binding of NAD has been examined by equilibrium dialysis utilizing [^{14}C]NAD in an effort to understand the overall enzyme mechanism.

An explanation of the results can be made on the basis of "sequential" changes (Conway and Koshland, 1968; Koshland *et al.*, 1966; Kirtley and Koshland, 1967) of subunit conformation induced by the binding of NAD, and indicate a combination of both positive and negative cooperative effects.

Experimental Section

Materials. GPD was purified from Red Star brand baker's yeast following the procedure of Krebs (1955). After four crystallizations, the enzyme exhibited an $OD_{280:260}$ ratio of 1.81–1.85. Charcoal treatment of the enzyme to remove any endogenous NAD was purposely omitted. The enzyme appeared to be homogeneous when tested by cellogel electrophoresis in Tris–borate buffer (pH 8.6) (Boyer *et al.*, 1963). Prior to equilibrium dialysis studies, crystals of the enzyme were centrifuged down, and dissolved in and dialyzed against 0.05 M sodium pyrophosphate buffer (pH 8.5), containing 0.001 M EDTA. Protein concentration was determined spectrophotometrically at

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[1] Abbreviations used are: GPD, glyceraldehyde 3-phosphate dehydrogenase; n_H, Hill coefficient; n_M, maximum number of binding sites, S_f, free substrate; S_B, bound substrate; $\bar{Y}$, fractional saturation; N_X, number of sites occupied by X per mole of enzyme; E_t, total enzyme. Intrinsic constant, K_i', is binding constant, K_i, corrected for statistical factors.

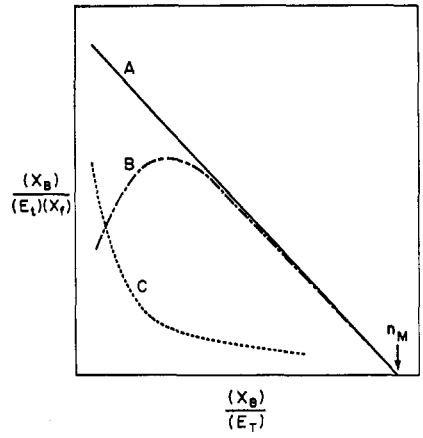

FIGURE 1: Hypothetical Scatchard plots. (A) Represents *n* independent and equal sites. (B) Represents *n* identical sites exhibiting positive cooperativity. (C) Represents either *n* identical sites exhibiting negative cooperativity or nonidentical binding sites without cooperativity.

280 mμ using a molar extinction coefficient determined by Krebs (1955) of 1.35×10^5 (corrected for a molecular weight of 145,000). In all experiments reported here, an enzyme preparation with a specific activity 200,000–220,000 was used. (One unit is defined as an increase of 0.001 at 340 mμ/min per mg of protein.) It has been found that the specific activity of the enzyme was somewhat dependent on the ionic conditions prior to or during assay. The precise specific activity therefore could not be taken as an index of enzyme purity. Electrofocusing experiments showed a single band with a minor shadow, indicating a single species. The enzyme was found to be stable for no longer than 3 months when stored as the crystalline suspension in ammonium sulfate containing EDTA (1 mM) and dithiothreitol (1 mM).

Equilibrium Dialysis. Equilibrium dialysis was routinely carried out in 0.3-ml cells at 3, 25, and 37°. Controls of NAD *vs.* buffer indicated that equilibrium was reached in 12 hr at 3 or 25° and in 5 hr at 37°. After equilibrium was reached ligand concentration was determined on aliquots from each cell compartment. Samples of 0.1 ml were counted in 10 ml of Bray's (1960) solution plus 1 ml of H_2O in a scintillation counter. The enzyme was checked periodically for any denaturation during experiments by using a standard reaction mixture which contained 50 μmoles of sodium pyrophosphate (pH 8.5), 10 μmoles of sodium arsenate, 0.468 μmole of glyceraldehyde 3-phosphate, and 0.650 μmole of NAD in a 1.2-ml total volume.

The yeast GPD used throughout this work was not subjected to charcoal treatment. The amount of NAD bound to the native enzyme was estimated from the OD_{280}:OD_{260} ratio by the method of Fox and Dandliker (1956) and by heat precipitation followed by a fluorometric determination of the released NAD (Lowry *et al.*, 1957). The results of the two methods were consistent and indicated less than 0.05 mole of NAD bound per mole of enzyme.

[^{14}C]NAD was prepared from [7-^{14}C]nicotinamide (New England Nuclear) by enzymatic exchange as described by Colowick and Kaplan (1957). The specific activity of the [^{14}C]NAD thus prepared was 77,538 cpm/μmole and found to be free of [^{14}C]nicotinamide by cellogel electrophoresis.

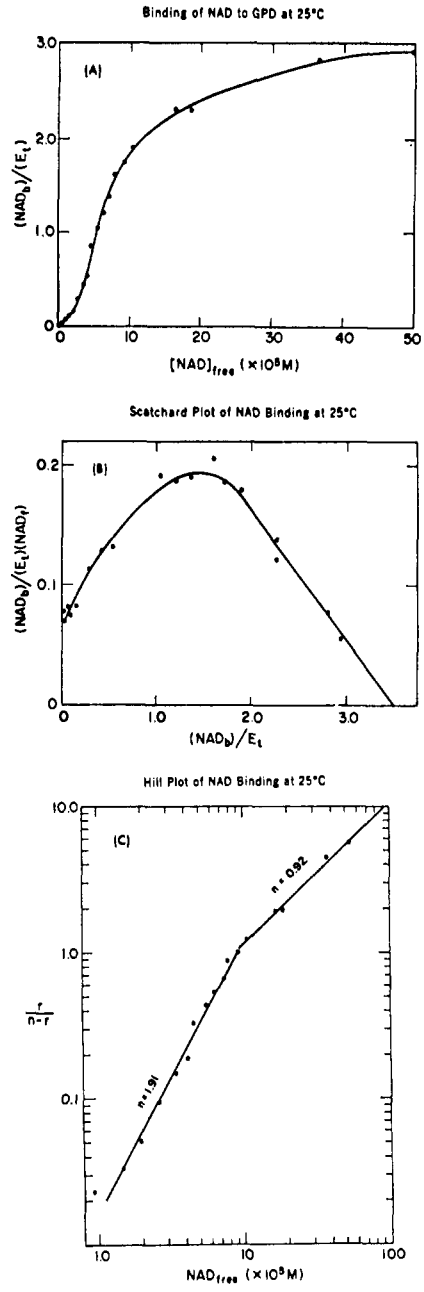

FIGURE 2: Equilibrium dialysis study of binding of NAD to yeast at 25°. Conditions: 0.05 M sodium pyrophosphate buffer, 1 mM EDTA (pH 8.5), 25°, and 8.0×10^{-5} M GPD. (A) $\bar{Y}$ *vs.* (S) plot, (B) Scatchard plot, and (C) Hill plot.

NAD was purchased from Boehringer und Sohne, Mannheim. Glyceraldehyde 3-phosphate diethylacetal barium salt was purchased from Sigma and converted into the free acid as described by Sigma.

Results

Binding Studies of NAD to Yeast GPD. The availability of large quantities of homogeneous GPD facilitated an examination of the binding of NAD to the enzyme by the method of equilibrium dialysis (Klotz, 1953; Scatchard *et al.*, 1957).

TABLE I: Summary of Results from NAD Binding Studies with Yeast Glyceraldehyde 3-Phosphate Dehydrogenase at Various Temperatures.

Temp (°C)	No. of Binding Sites[a]	Interaction Coefficients (n)[b] 10–50% Satn	50–90% Satn	K_{assocn}[c]
3–4	3.3	1.91	1.04	0.72×10^5
25–26	3.5	1.91	0.92	0.11×10^5
37	3.5[d]	1.61[d]	0.99[d]	0.035×10^5[d]

[a] Determined from extrapolation of linear portion of Scatchard plots. [b] Determined from the slopes of the Hill plots. [c] Determined from the slopes of the linear portions of the Scatchard plots. [d] 37° values are very rough since enzyme denatured appreciably during dialysis.

Plotting $(S_B)/(E_t)/(S_f)$ as a function of (S_f), where $(S_B)/(E_t)$ is the moles of ligand bound per mole of enzyme and S_f is the concentration of free ligand, a straight line is obtained if the sites are independent (Klotz, 1953). Deviation from a straight line is indicative of interaction between sites or dissimilarity of the sites. In Figure 1 the deviations to be expected for positive cooperativity, negative cooperativity and a mixture of polymorphic forms are shown.

Equilibrium dialysis with yeast GPD was carried out as previously described in the Experimental Section with the NAD concentrations varied over the range 0.1–10 K_m. A typical binding curve for NAD at 25° is presented in Figure 2A. The binding curve exhibits obvious sigmoidicity at low NAD concentrations and does not reach a maximum at high NAD concentrations. When the data were replotted in the $(S_B/E_t)/(S_f)$ *vs.* (S_f) plot a curve characteristic of positive cooperativity was observed (Figure 2B), but the extrapolated n_M value was only 3.5. The shape of the plot can be compared to Figure 1. Experiments attempted with different enzyme preparations and at several GPD concentrations in the region of 7–16 mg/ml gave similar results. There was no loss in enzyme activity during the duration of the equilibrium dialysis experiment as indicated by recovery of enzyme activity after the dialysis procedure was completed. Plotting the data of Figure 2A in a Hill plot gives n_H values of 1.91 below 50% saturation and 0.92 above 50% saturation (Figure 2C).

A typical binding curve for NAD at 4° is shown in Figure 3A. The shape of the curves are similar to those obtained at 25° but differ in the degree of cooperativity which increases with increasing temperature. The extrapolated n value from the equilibrium dialysis was 3.3 at 4° (Figure 3B) and the Hill plot again gave two n_H values (Figure 3C).

A summary of the Hill coefficients, the extrapolated n_M value from the Scatchard plots, and the K_{assocn} constant calculated from the linear portion of the Scatchard plot are given in Table I. Clearly the latter value is not a true constant but is included here to indicate in a rough way that

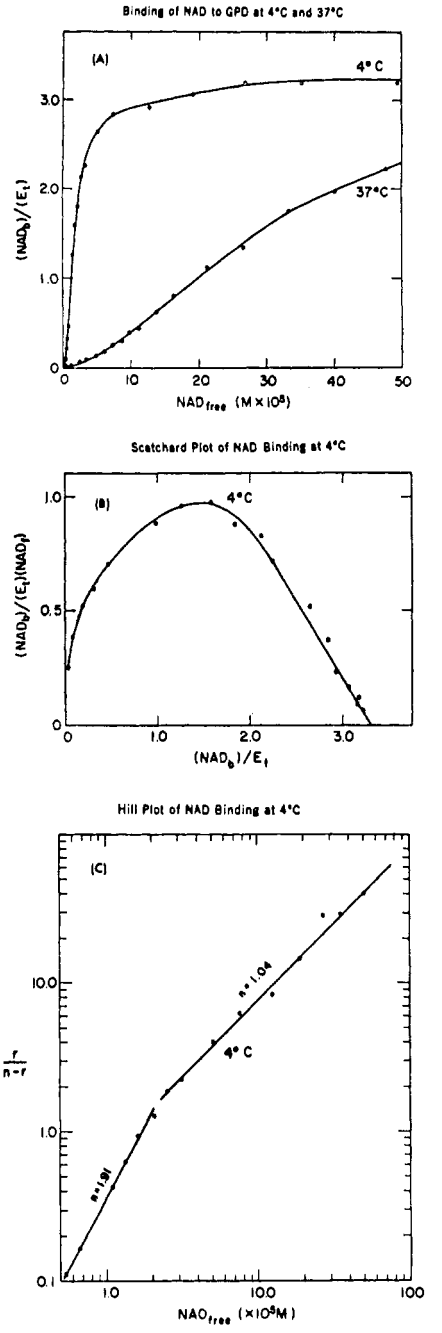

FIGURE 3: Equilibrium dialysis study of binding of NAD to yeast at 4 and 37°. Conditions: 0.05 M sodium pyrophosphate, 1 mM EDTA (pH 8.5), 8×10^{-5} M GPD at 4°, and 7.5×10^{-5} M GPD at 37°. (A) $\bar{Y}$ *vs.* (S) plot, (B) Scatchard plot at 4°, and (C) Hill plot at 4°.

the affinity of the enzyme at high NAD concentrations increases with temperature.

Discussion

The most striking features of the binding curves of the yeast enzyme are their deviations from conventional plots. The binding curves exhibit the deviations from the normal Michaelis–Menten equation and the two slopes

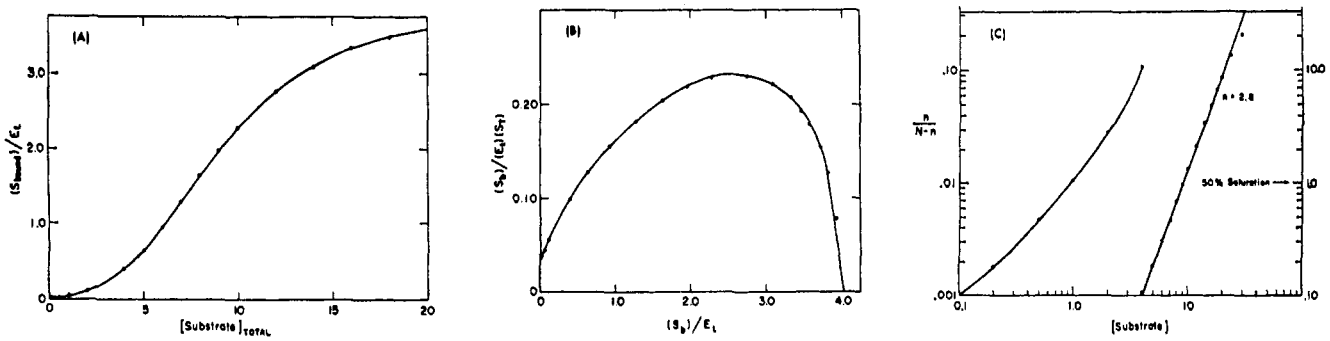

FIGURE 4: Theoretical sigmoid curve exhibiting positive cooperativity plotted as (A) $\bar{Y}$ *vs.* (S), (B) Scatchard plot, and (C) Hill plot. Oxygen binding data of Rossi-Fanelli *et al.* (1961) were fitted with a theoretical curve based on the simplest sequential model (Koshland *et al.*, 1966). The theoretical curve values which fit the oxygen binding data were then plotted in the three ways shown here. Hill plot is shown in two parts. Curve in middle range 10–90% is straight line with ordinate values on right. Curve in low range (1–10%) is curved line with ordinate values at left.

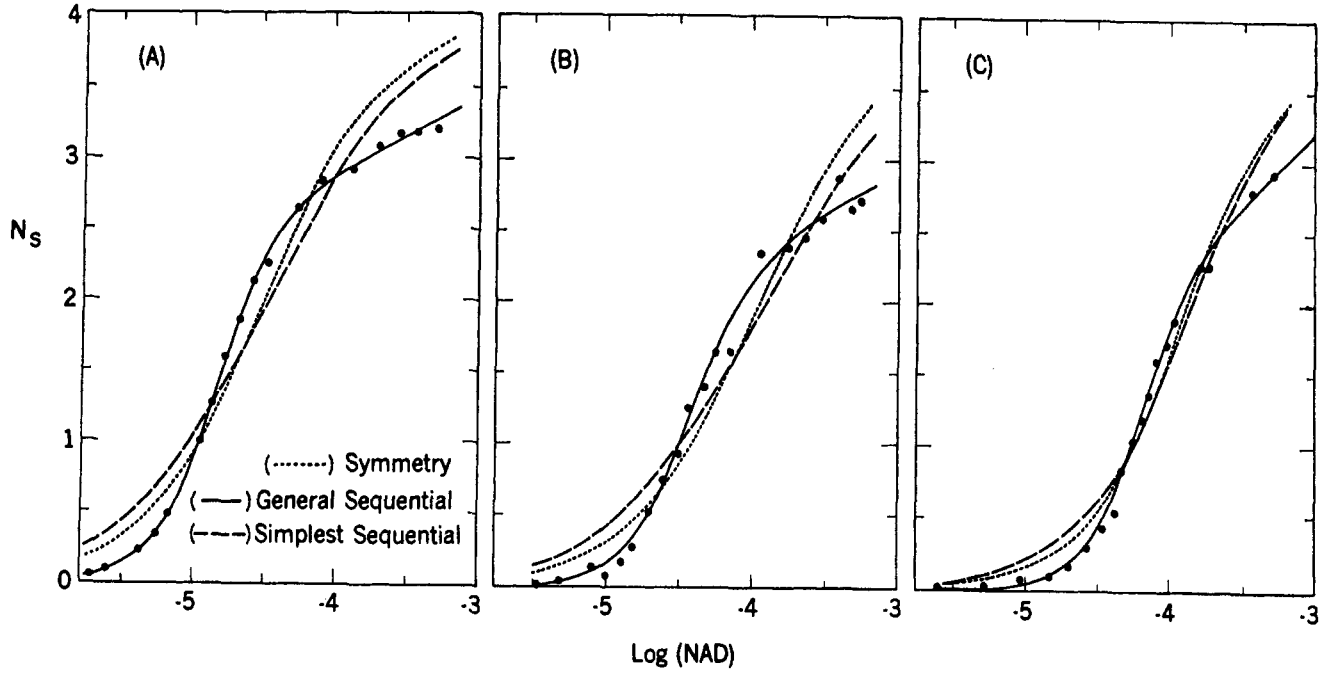

FIGURE 5: Comparison of theoretical curve and experimental data for binding of NAD to yeast GPD. Experimental data of one preparation at 4° (A) and two preparations at 25° (B and C). In each case computer program was asked to give the best fit using equations for general sequential model (intrinsic constants can assume any value), symmetry model with exclusive binding or nonexclusive binding ($K_2' < K_3' < K_4'$) and the simplest sequential model ($K_1'K_4' = K_2'K_3'$). Best fits are shown for general sequential (solid line), symmetry (dotted line), and simplest sequential (dashed line) models. Constants obtained for best fit listed in Table II.

in the Hill plots with a change in slope at approximately 50% saturation.

The unusual occurrence of two distinct n_H values for the one substrate, NAD, in both steady-state kinetic studies and equilibrium dialysis studies prompted an examination of the parameters associated with more conventional Hill plots. The theoretical curve (Koshland *et al.*, 1966) fitting the data of Rossi-Fanelli *et al.* (1961) on hemoglobin was plotted in a normal $\bar{Y}$ *vs.* (S) plot (Figure 4A) and the same curve was then plotted in the form of a Scatchard plot (Figure 4B) and a Hill plot (Figure 4C). The shape of the curve can be contrasted to those of the yeast enzyme. No break appears in the midpoint of the curve although, of course, there is a change from linearity at both extremes of the curve as is to be expected in a Hill plot. However, a change in slopes at 50% saturation is noticeably absent and a single n_H value of 2.8 is observed in the range 25–90% for hemoglobin data. The break in the NAD binding studies with yeast therefore appears to be a significant property of the yeast system rather than an anomaly of the Hill representation of the data. It is also significant that the hemoglobin data, although cooperative, extrapolate to a n_H value of 4.0 whereas the yeast GPD data extrapolate to values which are significantly less than 4.0.

Theoretical Interpretation. In order to determine the individual binding constants the binding data were fitted by

computer according to the procedure described previously (Cornish-Bowden and Koshland, 1970). The theoretical binding curve which gave the best fit to the data was found for the 4° data to have the intrinsic constants $K_1' = 4.5 \times 10^3$, $K_2' = 1.4 \times 10^5$, $K_3' = 7.5 \times 10^4$, and $K_4' = 3.6 \times 10^3$. These results suggest a mixture of positive and negative cooperativity in which the binding of the first molecule of NAD makes it easier for the next molecule to bind which in turn makes it more difficult for the third. The fit of these data is shown schematically in Figure 5A together with the best fits that could be obtained using the symmetry model (with either exclusive or nonexclusive binding) or the simplest sequential model (with either square or tetrahedral arrangements of subunits) either of these simpler models is capable of explaining the binding data for the yeast GPD. Moreover, other restrictions on the constants such as simple positive cooperativity, *i.e.*, $K_1' < K_2' < K_3' < K_4'$, and simple negative cooperativity, *i.e.*, $K_1' > K_2' > K_3' > K_4'$, did not fit the data any better than those shown schematically in Figure 5. Figures 5B and C show similar fits to 25° data for two preparations. The constants were slightly different for different experimental conditions but in each case the same result was obtained, *i.e.*, a mixture of negative and positive cooperativity in which K_1' is less than K_2', K_2' is greater than K_3', and K_3' is greater than K_4'. These data therefore would lead to the conclusion that the yeast enzyme must be explained by the general ligand-induced model in which the subunit interaction constants combine in a way to produce a mixture of positive and negative cooperativity. The best fit constants are shown in Table II.

TABLE II: Binding Constants for NAD to Yeast Glyceraldehyde 3-Phosphate Dehydrogenase.

Constants of Best Fit	Experimental Conditions: 4°	25° Prepn I	25° Prepn II
ψ constants of Adair equation[a]			
ψ_1	1.8×10^4	3.3×10^3	Not determined[b]
ψ_2	3.8×10^9	7.4×10^8	2.19×10^8
ψ_3	1.9×10^{14}	4.2×10^{12}	1.1×10^{12}
ψ_4	1.7×10^{17}	2.6×10^{14}	6.3×10^{14}
Intrinsic constants			
K_1'	4.5×10^3	0.83×10^3	Not determined[b]
K_2'	1.4×10^5	1.5×10^5	Not determined[b]
K_3'	7.5×10^4	8.5×10^3	7.3×10^3
K_4'	3.6×10^3	2.5×10^2	2.4×10^3

[a] Adair equation expressed in form

$$N_S = \frac{\psi_1(S) + 2\psi_2(S)^2 + 3\psi_3(S)^3 + 4\psi_4(S)^4}{1 + \psi_1(S) + \psi_2(S)^2 + \psi_3(S)^3 + \psi_4(S)^4}$$

[b] The fit in this experiment showed a ψ_1 value of 5.456 ± 1534. Since the error in this value is so large, the calculation of K_1' and K_2' values has little meaning.

The low K_3' and K_4' values obtained for the yeast GPD may be the explanation for obtaining n_M values of 3.2 to 3.5 instead of 4 by extrapolation in the equilibrium dialysis experiments. The observed difficulty in saturating the fourth site despite high NAD concentrations is most probably due to this negative cooperative effect and may be the reason why other enzymes never reach theoretical maximum saturation.

An alternative explanation of the results could be that there are two forms of the enzyme both of them cooperative with different midpoints or one cooperative and the other Michaelis–Menten. Computer simulations of such models do not give as good fit to the data as that observed in Figure 5 but the results are not so far off that this alternative can be excluded by the binding data alone. A number of sources have indicated that the yeast enzyme crystallized by the procedure described above is a single homogeneous protein containing a single type of subunit (S. Velick, 1969, personal communication). In our laboratory electrofocusing experiments have produced a single band of the protein and of the individual subunits in urea (W. Stallcup and D. E. Koshland, Jr., in preparation). Kirschner and Voight (1968) however have suggested that even these tests may not be sufficient to separate pure proteins and they have found separate bands for the yeast enzyme on polyacrylamide gel where single bands were found in electrofocusing experiments. The two bands isolated from the crystallization procedure described in their experiments however were functionally identical even though there appeared to be some differences which caused them to migrate differently under electrophoresis. Thus, there is at present no evidence that more than one functional form of the enzyme is present in these preparations of the yeast enzyme. However, it is extremely difficult to exclude the possibility of a minor amino acid replacement which would escape detection.

It is also possible that the enzyme crystallization procedure could produce some denatured protein which fails to bind NAD. In that case the calculations assuming totally active protein would lead to a deceptive value for sites occupied and a greater complexity to the intrinsic binding constants than might be expected. A moderately good fit (sum of squares of errors = 0.0116 compared to sum of squares of errors = 0.0189 for fits of Figure 5) of the binding data for one case can be obtained assuming the denatured protein comprises 16% of the protein and that it binds no NAD. Preparing denatured dehydrogenase by long standing in ammonium sulfate gave a protein with 20% of the specific activity of native enzyme which binds as well as native protein. Other attempts to fit the data assuming mixtures of native and denatured forms give errors far greater than this. Thus, it is not yet proved definitively that a single homogeneous enzyme has been obtained but the best fit from the mathematical standpoint is obtained with such an assumption and no other combination has yet been shown to be in as good agreement with all the facts.

It is inevitable to compare these results and conclusions derived from them to those of Kirschner *et al.* (1966) using temperature-jump measurements. There are some notable points of agreement in the observation of sigmoid binding at 37° and the fact that the sigmoidicity increases on going from 25 to 37°. However, we observe a distinct

sigmoid saturation curve at 25° whereas they report Michaelis–Menten kinetics. Our binding data at 37° are not sufficient to allow one to draw definitive conclusions at that temperature and yet the similarity of the present results at 4, 25, and 37° suggest that the pattern at all of these temperatures is similar. There are significant differences however in the two enzyme preparations. The yeast strains are different in the first place. Our enzyme was not treated with charcoal whereas their enzyme was. Our most reliable data were obtained at 25 and 4° whereas their studies were essentially applied to the enzyme at 40°. Charcoal treatment of the rabbit muscle enzyme tended to blur differences in the binding constants (Conway and Koshland, 1968). The same effect on the yeast enzyme which is much less cooperative to begin with could conceivably produce a binding curve close to that of Michaelis–Menten at 25°. The different deductions from the two techniques however suggest that further careful analysis of these systems will lead to new insight on protein interactions. Collaborative studies to this effect are now under way.

Considering all the evidence it would seem that yeast GPD is composed of a single fundamental type of subunit and binds NAD with a mixture of positive and negative cooperativity. If it is a homogeneous protein such a binding pattern requires the general ligand-induced model. As such it provides a new example of cooperative interactions. Hemoglobin shows a simple positive cooperativity. CTP synthetase shows positive cooperativity with some ligands and negative cooperativity with others. Rabbit muscle GPD shows simple negative cooperativity. In the case of the yeast enzyme a single ligand shows a mixture of positive and negative cooperativity.

Positive cooperativity has been presumed to show selective advantages by making the enzyme more responsive to environmental changes and negative cooperativity by insulating the enzyme against metabolic fluctuations. One may well ask the advantage of a mixture of these two features. Possibly this behavior provides added sensitivity over some ranges of concentration and decreased sensitivity over another. However in this case the fluctuations in the binding constants yield a curve quantitatively similar to a Michaelis–Menten curve. It is conceivable in this case that the positive and negative cooperativity is not of major importance as far as the kinetics of this enzyme are concerned. Its importance may lie in the indication that all permutations in subunit interactions are possible and this range of possibilities is what is vital to the living system. Not every protein needs all 20 amino acids, but nature needs the variety provided by having 20 amino acids to design many different proteins. Similarly not every protein requires cooperativity but the potential for cooperative interactions must be preserved, and this means a variation in subunit interaction energies. When no cooperativity is required, the K_{AB}, K_{BB}, etc., values may equal 1 thus leading to a Michaelis–Menten curve. However, it may also happen that the values do not precisely equal 1 in which case cooperativity is observed but the kinetic consequences are only minor deviations as far as the biological system is concerned.

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