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Malate Dehydrogenase, Anticooperative NADH, and L-Malate Binding in Ternary Complexes with Supernatant Pig Heart Enzyme†

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ABSTRACT: Supernatant malate dehydrogenase from pig heart, a dimeric protein containing two very similar or identical subunits, shows negatively cooperative (anticooperative) interactions between NADH binding sites in the presence, but not in the absence, of 0.1 *M* L-malate. This behavior is observed consistently whether the technique used employs protein fluorescence quenching, NADH fluorescence enhancement, or ultrafiltration dialysis. Fluorescence titration shows that L-malate is also anticooperatively bound in the presence of saturating concentrations of NADH. The data are consistent with an "induced asymmetry" model in which conformational change accompanies the formation of the ternary complex. Two of the three chromatographically resolvable forms of the enzyme have been tested and found to have anticooperative behavior.

Although the biological significance of heteropolymeric enzyme structure is now better understood in terms of regulatory function, the significance of homopolymeric enzyme function is still in question. The near ubiquitous occurrence of multiple catalytic subunits in heteropolymeric as well as homopolymeric enzymes suggests a strong evolutionary selection for as yet poorly understood functional advantages. The importance of protein conformational alteration has been established as a significant feature in heteropolymeric proteins, suggesting that conformational mobility also may be important in homopolymeric enzyme function. One consequence of conformational change related to catalytic mechanism might be negatively cooperative (anticooperative) interactions between ligand binding sites on enzyme multimers. The prediction that negatively cooperative interactions might occur (Koshland et al., 1966) has been followed by experimental reports (Malhotra and Bernhard, 1968, 1973; Levitzki and Koshland, 1969, 1972; Simpson and Vallee, 1970; Long et al., 1970; Lazdunski et al., 1971; Levitzki et al., 1971; Dalziel and Egan, 1972) of this sort of binding behavior.

We report here studies aimed at exposing possible anticooperative interactions in pig heart supernatant malate dehydrogenase. These studies were prompted by the fact that such behavior would be predicted in mitochondrial malate dehydrogenase by the previously published studies of Harada and Wolfe (1968) described in a reciprocating compulsory order kinetic mechanism which assumed functional interdependence between enzyme subunits. Previous attempts to discover anticooperative binding of ligands by this and the mitochondrial enzyme (Holbrook and Wolfe, 1972) were apparently unsuccessful because of subtle dependence of the phenomenon on the structure of the substrate analog used in binding studies.

Materials and Methods

Reagents. Pig heart cytoplasmic malate dehydrogenase (s-MDH)[1] was prepared by a modification (Eberhardt, 1972) of the method of Gerding and Wolfe (1969). The enzyme had a specific activity of 100 IU/mg of protein using $A_{280}(1\%) = 9.0$ (Gerding and Wolfe, 1969). All binding data reported here were taken with the use of the second s-MDH peak eluting from hydroxylapatite column chromatography in the final purification step with one exception which will be considered below. The crystalline enzyme was

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[1] Abbreviation used is: s-MDH, supernatant or cytoplasmic malate dehydrogenase.

recovered from $(NH_4)_2SO_4$ suspension by centrifugation and dialyzed against pH 8.0 Tris-acetate which was 0.5 *M* with respect to acetate.

Sigma grade III NADH was purified immediately before use by column chromatography on DEAE-cellulose. Elution was accomplished from a 1.5 × 50 cm column with a 300-ml 0.15–0.25 *M* NH_4HCO_3 linear gradient. The purified NADH, which had an absorbance ratio (260/340 nm) of 2.3, was used in the NH_4HCO_3 solution in titration. Coenzyme so specified has been found to react quantitatively in enzyme-catalyzed reactions. NADH concentrations were determined assuming an ϵ_{340} of 6.22×10^3. Reagent grade L-malate was obtained from Sigma and used without further purification. Sigma Tris primary standard was used to prepare the buffer.

Instrumentation. Fluorescence spectra and fluorescence binding studies were all made with a Schoeffel split beam spectrofluorometer equipped with two monochromators for exciting light and a single monochromator for the fluorescence emission. The spectral wavelength from 300 to 550 nm was continuously scanned at slow speed with the excitation at 290 nm in the determination of emission spectra.

Ligand titration fluorescence measurements were also made with the Schoeffel spectrofluorimeter interfaced with the Varian 620/i computer to facilitate signal averaging, over a 10-nm wavelength range, and data manipulation. Data treatment by boxcar integration involved averaging 1000 observations over a 20-sec time period. Measurements were repeated after each addition of titrant until the measured values stabilized.

Binding Measurements. The amount of NADH bound in fluorescence binding studies was inferred either by measuring the NADH fluorescence enhancement (fluorescence of bound less fluorescence of an equivalent amount of free NADH) at 410 nm or protein fluorescence quenching at 350 nm with excitation at 290 nm in both cases. Alternatively, NADH fluorescence enhancement was measured at 440 nm with excitation at 340 nm. Except for the ultrafiltration binding measurements at 27°, all spectra and binding measurements were made at 25° in pH 8.0 Tris-acetate buffer which was 0.5 *M* with respect to acetate.

The enzyme was titrated in a 3.5 ml volume contained in a 1.0 × 1.0 cm quartz cuvet by the addition of 10-μl quantities of titrant from a microsyringe. The titrant ligand concentration was adjusted to about 30–50 times that of the site concentration of the enzyme unless ligand was very loosely bound (i.e., L-malate), in which case the higher concentrations given in the figures were used. Titrations of NADH into s-MDH, NADH into s-MDH containing 0.1 *M* L-malate, and L-malate into s-MDH saturated with NADH were done under one or more of the physical conditions described in the paragraph above. The titrant ligand solution contained all solutes, including enzyme, at concentrations present in the enzyme solution being titrated. For example, in the titration of NADH into enzyme in the presence of L-malate, the titrant NADH contained enzyme, L-malate, and buffer at the same concentration as that in the enzyme solution being titrated in the cuvet. This procedure eliminated troublesome dilution corrections in the analysis of data. Fluorescence enhancement was measured by subtracting an NADH "blank" from a similar titration curve measured in the presence of enzyme.

Ultrafiltration binding studies were carried out with the use of a Metalloglass ultrafiltration cell equipped with Visking dialysis tubing as filter membrane. Volumes of 0.6 ml of equilibrated s-MDH (1.0 μM), NADH (at appropriate concentrations up to 20 μM), and L-malate (0.1 *M*) were added to each of the eight channels in the ultrafiltration apparatus. About 0.2 ml of the solution was forced through the membrane under nitrogen pressure of about 32 psi. The concentration of the NADH passing through the membrane was then determined in the 0.2-ml sample of ultrafiltrate by measuring the fluorescence in a Hatachi MFP-2A spectrofluorimeter with excitation at 340 nm and emission measured at 440 nm. The NADH concentration was established by comparison with a standard curve of fluorescence vs. NADH concentration prepared with the use of NADH which had been purified as described above under Reagents. The small increase in protein concentration with ultrafiltration was ignored.

As subunit dissociation might account for the inability to observe site interactions, the enzyme was examined by gel filtration under experimental conditions approximating those used in the binding titrations. The column gel filtration behavior of the enzyme was examined in the presence of NADH (150 μM), in the presence of both NADH (150 μM), and L-malate (100 m*M*) and in the absence of these ligands.

Data Analysis. Correction was made for background fluorescence by subtracting the blank from the sample fluorescence value. Since the NADH bound by enzyme reduces the concentration of free NADH, particularly at high enzyme concentration, the concentration of free NADH was calculated from the following relationship:

$$[\text{NADH}]_{\text{free}} = [\text{NADH}]_{\text{total}} - [\text{NADH}]_{\text{bound}}$$

$$[\text{NADH}]_{\text{free}} = [\text{NADH}]_{\text{total}} - \frac{\Delta F}{\Delta F_{\text{sat.}}}\,(\text{enzyme site})$$

The $\Delta F_{\text{sat.}}$ in the above equation is the total fluorescence increment at ligand saturation of binding sites and it is most conveniently determined by appropriate linear extrapolation of a double reciprocal plot of $1/(\text{NADH})_{\text{total}}$ vs. $1/\Delta F$. Because errors from this source are less significant at lower enzyme concentrations, it is desirable to titrate enzyme at concentrations less than the value of the ligand dissociation constant. The use of this correction for bound NADH and the accuracy of the extrapolated fluorescence enhancement or quench with ligand binding are relatively critical factors in the precision of the fluorescence binding studies.

The ultrafiltration dialysis data were treated assuming no significant Donnan effect under the experimental conditions used. It is apparent that this alternative technique avoids some of the technical problems and possible artifacts unique to the direct fluorescence titration method.

The most precise data, that for L-malate binding in the presence of saturating NADH (Figure 7) and that for NADH binding in the presence of 0.15 *M* L-malate (Figure 4), were analyzed for fit to the sum of two hyperbolas using the method described by Cleland (1968). These derived values for the first and second binding constants (see Table I) may be compared with values calculated from the Hill plots which were calculated as described below.

Results

Fluorescence titration spectra showing the changes in fluorescence as a function of the amount of NADH bound by s-MDH and the same in the presence of saturating L-malate are shown in Figure 1A and B, respectively. It is apparent that NADH binding, in the presence or in the absence

Table I: Calculated Association Constants for Supernatant Malate Dehydrogenase.[a]

Ligand	n_H	$X_{0.5}$ (M)	Association Constants (μM^{-1})			
			Induced Model		Preexisting Asymmetry Model	
			K_1	K_2	K_1	K_2
NADH	0.97	8.7×10^{-7}	1.08	1.22	1.62	0.81
NADH in presence of 0.15 M L-malate	0.63	6.7×10^{-7}	3.24 [12.5 ± 0.94]	0.68 [1.09 ± 0.13]	6.0	0.37
L-Malate in the presence of 150 μM NADH	0.65	2.7×10^{-3}	802 [1330]	170 [110]	1481	92

[a] Summary of the values of association constants calculated assuming the two models discussed in the text. Values for the constants are calculated from Hill plot data for NADH binding (Figure 3), NADH binding in the presence of saturating L-malate (Figure 4), and for L-malate binding in the presence of saturating NADH (Figure 7). Values in brackets are derived from best fit to two hyperbolae rather than from the Hill plot as described in the text.

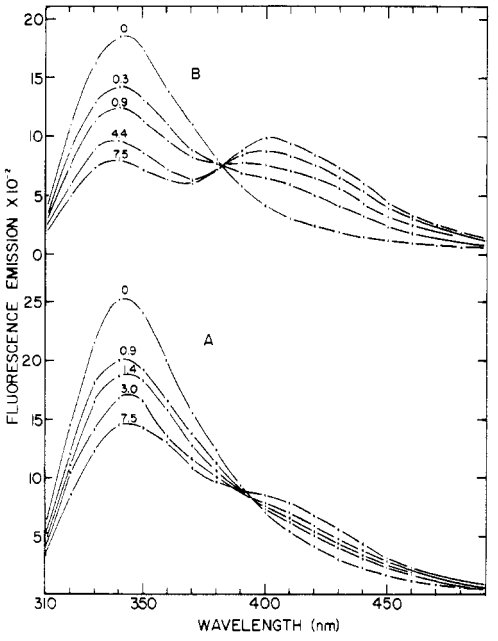


FIGURE 1: Fluorescence emission spectra of 1 μM supernatant malate dehydrogenase with activation at 290 nm. The numbers by each scan indicate the concentration of NADH (free and bound by enzyme) in μM. Curves in A and B represent titrations in the absence and presence, respectively, of 0.1 M L-malate corrected for free NADH fluorescence. Physical conditions and instrumentation for this and the following figures are explained under Materials and Methods.

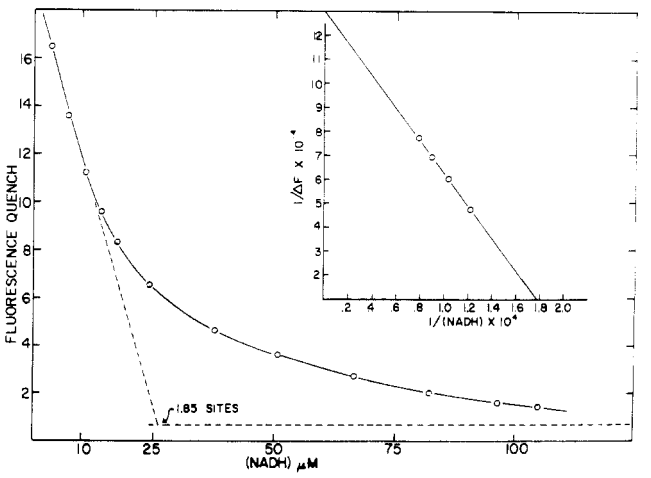


FIGURE 2: Fluorescence titration of 14 μM s-MDH with NADH. Excitation was at 290 nm and emission was measured at 350 nm. Because enzyme sites were initially much greater than NADH in concentration and the NADH binding is very tight, no corrections for unbound NADH have been applied to these data.

of L-malate, is accompanied by about the same amount of quench in the protein fluorescence at about 343 nm. It is also apparent that the natural fluorescence of NADH, at wavelengths between 390 and 450 nm, is enhanced by binding to the enzyme (Figure 1A) and that this is further enhanced by NADH binding to the enzyme in the presence of L-malate (Figure 1B). There is an isoemissive point at about 395 nm in the case of the enzyme–NADH complex which shifts to about 382 nm in the enzyme–NADH–L-malate complex. The occurrence of isoemissive points in these spectra suggests a relatively simple change in the fluor environment and the formation of a single fluorometrically distinguishable species with ligand binding. The protein fluorescence quench and the NADH fluorescence enhancement signals are therefore useful probes in the study of ligand binding by the enzyme.

A stoichiometry of two NADH molecules bound per s-MDH dimer is supported in the data of Figure 2. At high enzyme concentration (14 μM) the initial slope of the enzyme titration curve extrapolates to 1.85 sites per enzyme dimer in good agreement with data published by Pfleiderer and Auricchio (1964), Thorne and Kaplan (1963), Fisher et al. (1969), and Holbrook and Wolfe (1972). The proportionality between the fluorescence quench and NADH binding by s-MDH throughout the entire titration of two ligand binding sites is supported by the data in Figure 2. It is apparent from the double reciprocal plot (Figure 2, inset) that linear behavior, consistent with simple proportionality between ligand binding and fluorescence quench, holds up to about 95% saturation of the dimer with NADH. Data points for the several highest NADH concentrations only have been included in the inset diagram.

The Hill plot illustrated in Figure 3 has a slope of 0.97, a value indicative of simple hyperbolic binding and the equivalence of binding sites. The binding curve is apparently a rectangular hyperbola (inset B) as expected if the binding sites have no discernible interaction. The double reciprocal plot (inset A) is approximately linear as expected, if no site interaction occurs.

Figure 4 illustrates the titration of 0.1 μM s-MDH with NADH in the presence of 0.15 M (saturating) L-malate. It is apparent from the Figure 4 insets that the binding is not that of a rectangular hyperbola, particularly in the nonlin-

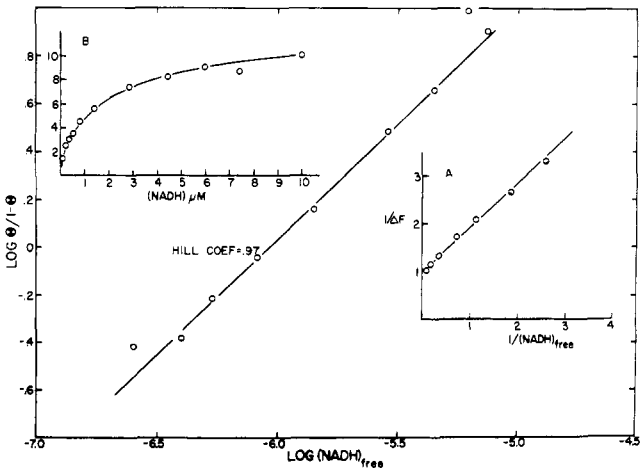


FIGURE 3: Hill plot and fluorescence titration data for the titration of 0.1 μM supernatant malate dehydrogenase. Excitation was at 290 nm and emission was measured at 350 nm. The linear double reciprocal plot (inset A) and the approximate rectangular binding curve (inset B) as well as the Hill coefficient approximating 1.0 are all consistent with independent binding of NADH at both sites in the enzyme dimer. The symbol θ represents the fraction bound. The ordinate in inset B represents fluorescence enhancement in arbitrary units.

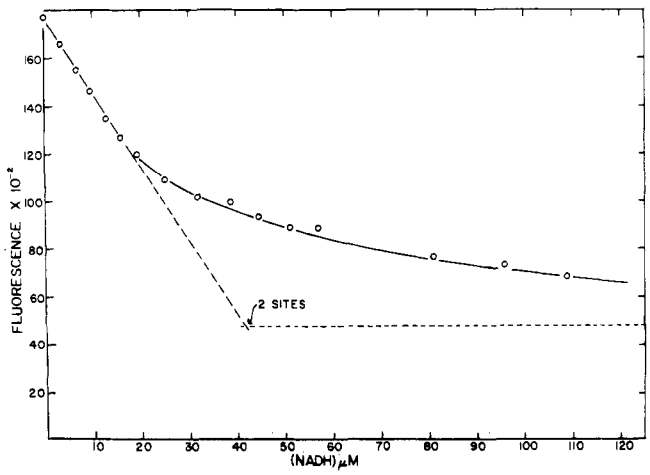


FIGURE 5: Fluorescence (quench) binding curve for 20 μM s-MDH in the presence of 0.1 M L-malate. Excitation was at 290 nm and emission was measured at 350 nm. Extrapolation of initial binding slope to the saturation fluorescence value (horizontal dashed line) yields the number of coenzyme binding sites paralleling the experimental data presented in Figure 2.

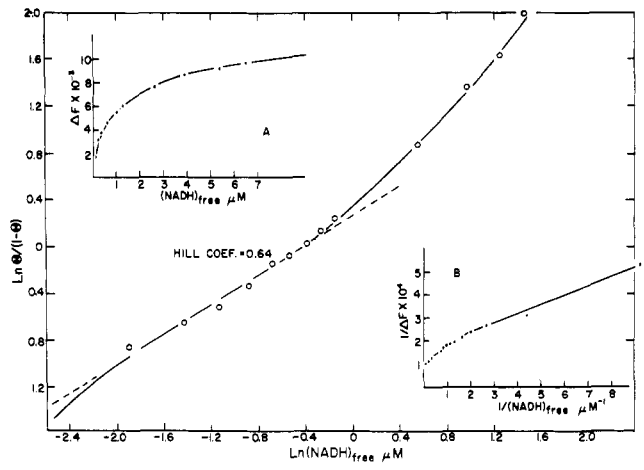


FIGURE 4: Hill plot and fluorescence titration of 0.1 μM s-MDH in the presence of 0.1 M L-malate. Excitation was at 290 nm and emission was measured at 350 nm. The nonlinear double reciprocal plot (inset B) indicates that binding is not rectangular hyperbolic in form. The Hill coefficient of 0.64 is consistent with anticooperative interactions between the two coenzyme binding sites.

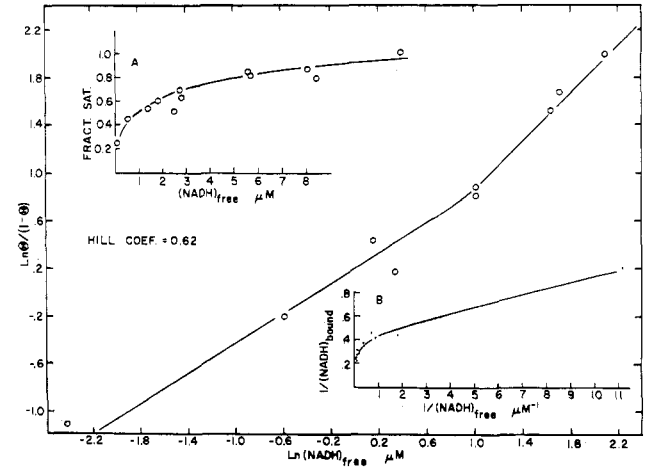


FIGURE 6: Hill plot and ultrafiltration binding titration data for 4 μM s-MDH (sites) in the presence of 0.1 M L-malate at 27°. The nonlinear double reciprocal plot (inset B) indicates that binding does not have the form of a rectangular hyperbola (inset A) consistent with anticooperative interactions between the NADH binding sites.

ear double reciprocal plot (inset B), in which the downward curvature at high NADH concentration is consistent with anticooperative behavior. The main graph in Figure 4 is a Hill plot of the same data with a slope of about 0.64 at half-saturation of the dimer ($\ln \theta/(1-\theta) = 0$). This slope is consistent with anticooperative interactions between the two NADH binding sites.

The titration of enzyme at high concentration (20 μM) with NADH in the presence of L-malate is shown in Figure 5. Again it is apparent that two sites are involved (by extrapolation of the initial slope to fluorescence quench saturation). Since the intercept of the extrapolated initial slope with the saturation quench value (horizontal dashed line) at high concentration of the enzyme gives the known stoichiometry for NADH binding and the reciprocal fluorescence vs. reciprocal NADH concentration curve (not shown but similar to Figure 3) is linear at concentrations approaching site saturation, this suggests linear proportionality in the fluorescence quench and ligand binding for both ligand binding sites. Other evidence supporting this conclusion will be given below.

As a precaution against artifact in the fluorescence titration technique, the ligand titration was repeated using the NADH fluorescence enhancement signal (410-nm emission) to indicate binding. The result was qualitatively and quantitatively the same as that shown in Figure 4. The Hill plot had a slope of about 0.63 and the double reciprocal plot curved downward at high NADH (low 1/NADH) concentration.

As an independent check against artifact in the fluorescence titration, equilibrium ultrafiltration experiments were carried out. The data are presented in Figure 6. Again the results support anticooperative interactions between the two NADH binding sites and a Hill plot slope of 0.62 in agreement with the fluorescence titrations.

The apparent dependence of NADH binding anticooperativity on the binding of L-malate led to the titration of L-

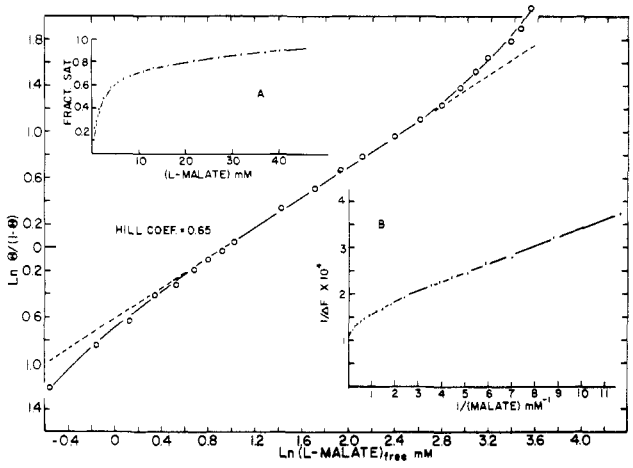


FIGURE 7: Hill plot and fluorescence titration of L-malate binding in the presence of 1.0 μM s-MDH and 150 μM NADH with excitation at 290 nm and emission measured at 410 nm. Anticooperative L-malate binding is indicated by the nonlinear double reciprocal plot (inset B), apparent binding which is not in the form of a rectangular hyperbola (inset A), and the Hill coefficient of less than one.

malate into enzyme saturated with NADH. Figure 7 shows that L-malate is itself anticooperatively bound on the s-MDH dimer in the presence of saturating NADH. Although the stoichiometry of L-malate binding cannot be determined because of its relatively large dissociation constant, it seems quite reasonable to assume behavior parallel to that observed in NADH binding, namely that anticooperative interactions occur between two L-malate binding sites on the enzyme.

Gel filtration studies give no discernible evidence that the enzyme dimer is measurably dissociated, in the presence or absence of the ligands, under any of the experimental conditions tested. The elution volume of unliganded enzyme, enzyme with NADH, and enzyme with both NADH and L-malate all elute with bovine serum albumin, a protein of very similar molecular weight which is known not to be dissociated under the experimental conditions used.

It has not been possible to determine whether enzyme half-saturated with L-malate binds NADH in an anticooperative manner although nonlinear double reciprocal plots are obtained.

Preliminary experiments indicate that association constants for NADH are sensitive to ionic strength. Ionic strength might be an important factor in the binding constants for L-malate but this problem has not been studied in detail.

The highest precision data were evaluated for best fit to two hyperbolae as described in the Materials and Methods section. The derived values of the two binding constants are listed in brackets in Table I for comparison with similar values obtained from the Hill plots of data as described in the following Discussion. The NADH binding constants were derived from data presented in Figure 4 (inset A) and the L-malate binding constants were evaluated from data presented in Figure 7 (inset A). The differences between the two sets of values in Table I are accounted for by the inherent inaccuracy of the evaluation methods used.

Discussion

Ligand binding studies with the use of a filter fluorometer by Holbrook (1972) and Holbrook et al. (1972) have shown protein quench of several dehydrogenases not to be linearly proportional to NADH binding sites occupied under their experimental conditions. We therefore took precautions, in measurements with the use of a monochromator-equipped spectrofluorometer, to observe and avoid possible artifact from this source. We believe fluorescence changes observed under our experimental conditions to be linearly proportional to ligand site occupancy whether NADH fluorescence enhancement or protein fluorescence quench is being observed. Several observations support this belief. As shown in Figures 1A and B, the presence of an isoemissive point in the fluorescence emission spectra in the presence or in the absence of 0.1 M L-malate indicates that we are observing relatively simple changes in the fluor environment with ligand binding whether one or both subunits are occupied with NADH or NADH and L-malate. Further, consistent results, yielding almost identical Hill coefficients, are obtained for NADH binding in the presence of 0.1 M L-malate by both fluorescence and ultrafiltration techniques (Figures 4 and 6). Also, as shown above, the subunits do not dissociate to a measurable extent in gel filtration studies. Therefore, we interpret the observation that NADH binding in the absence of L-malate gives linear double reciprocal plots and a Hill plot slope of about one to indicate little or no site interaction and a linear proportionality between fluorescence change and NADH binding sites occupied. Since, except for the presence of L-malate, the same physical conditions prevail when NADH binding is observed in the presence of L-malate, it seems reasonable to interpret the nonlinear double reciprocal plot (Figure 4, inset B) for NADH binding in the presence of L-malate in terms of anticooperative binding site interactions. The possibility that L-malate somehow induces a change in the physical properties of the protein resulting in nonlinear proportionality between fluorescence change and ligand binding seems remote.

It is of interest that anticooperative NADH binding behavior is apparently induced with the formation of the enzyme-NADH-L-malate ternary complex. This might be explained by the induction of a conformational change with ternary complex formation. This interpretation is correlated with the observation by Frieden and Fernandez Sousa (1975) that the "NADH on" rate approximates the diffusion-limited rate whereas the "malate on" rate is much slower than that predicted for a diffusion limited reaction. Other studies (Eberhard and Wolfe, 1975), which used circular dichroism difference spectra to study ligand binding, may be interpreted to indicate that the nature of subunit interactions are dependent upon the molecular structure of the substrate analog ligands. This interpretation is consistent with the "induced fit" hypothesis proposed by Koshland et al. (1966). It is also of interest that supernatant malate dehydrogenase has been found to form an asymmetric molecular form which binds one NAD^+ per dimer in crystallographic studies by Tsernglou et al. (1972).

The anticooperative binding behavior described here applies to the middle of three enzymatically active, chromatographically resolved, homogeneous fractions eluting in hydroxylapatite chromatography. Studies of the first enzyme component eluting from this column chromatography indicate that it also has anticooperative binding behavior. If one assumes that the three chromatographic forms of the enzyme represent a binomial distribution with two structurally differing subunits, at least one of the chromatographic components studied here would be predicted to be a strictly homopolymeric form of the enzyme. This follows if the ex-

pressed chromatographic properties of the hybrid are the average of the two "thoroughbred" (precisely homopolymeric) forms of the enzyme.

As a dimeric protein with two catalytic sites, supernatant malate dehydrogenase is particularly attractive to test for fit to theoretical models. We conceive of only two models consistent with the data. These are considered below.

The binding of ligands to a two-subunit enzyme may be easily quantitated using the midpoint and slope of the Hill plot (F. W. Dahlquist and D. E. Koshland, Jr., paper in preparation, 1975). The binding of the ligand X can be represented by an Adair equation

$$N_X = \frac{(\psi_1 X + 2\psi_2 X^2)}{(1 + \psi_1 X + \psi_2 X^2)} \quad (1)$$

where N_X is the moles of ligand bound per mole of enzyme dimer and ψ_1 and ψ_2 correspond to the phenomenological formation constants for the singly and doubly bound enzyme species, respectively. The midpoint of the Hill plot, $X_{0.5}$, is given by

$$X_{0.5} = \left(\frac{1}{\psi_2}\right)^{1/2} \quad (2)$$

and the Hill coefficient, η_H, evaluated at the midpoint is given by

$$\eta_H = \frac{4}{[\psi_1/(\psi_2)^{1/2}] + 2} \quad (3)$$

There are two alternative microscopic explanations for apparent anticooperativity in ligand binding which affect the microscopic interpretations of the Adair constants.[2] One explanation supposes that the binding sites on each subunit are identical and that the apparent negative cooperativity in ligand binding results from interactions induced between the binding sites with ligand binding. Under these conditions $\psi_1 = 2K_1$ where K_1 is the microscopic formation constant for the binding of ligand to either of the two identical sites. The alternative to this explanation supposes that despite the fact that both subunits have identical amino acid sequences, the architecture of the dimeric molecule imposes an asymmetry in the binding sites such that the ligand-free binding sites are nonidentical. For such a scheme, the apparent anticooperativity is actually the result of the different affinities for the two sites and interactions between sites do not occur. For this scheme, $\psi_1 = K_1 + K_1'$ where K_1 and K_1' refer to the formation constants for ligand binding to each of the two sites. In both cases (induced and preexistent asymmetry), the second Adair constant is simply the product of each microscopic constant. Thus ψ_2 is equal to K_1K_2 in the induced model and is equal to K_1K_1' in the preexistent asymmetry model. Since both models predict identical Adair binding equations, they cannot be rigorously distinguished by binding data alone. Table I shows the alternative microscopic interpretations of the binding data described here.

The Hill coefficient for NADH binding in the absence of L-malate has a calculated value of 0.97 (Figure 3) and a midpoint value of 8.7×10^{-7} M. This value, within the limits of error, is consistent with simple Michaelian binding behavior. However, the sensitivity with which asymmetry can be detected depends in part upon the mechanism. For example, one may calculate from the experimentally determined Hill coefficient (assuming the value 0.97 to be significant for purposes of discussion) and the midpoint, affinity constant values of 1.08 and 1.22×10^6 M^{-1} for the induced model; and values of 1.62 and 0.82×10^6 M^{-1}, assuming the preexistent asymmetry model. The latter values are strongly dependent upon the value of the Hill coefficient and an error of 0.01 in its value would change the ratio of the affinity constant values drastically. It follows that the nearly hyperbolic behavior observed cannot unequivocally rule out preexistent asymmetry in the enzyme and that relatively large differences in the affinities at the two distinct ligand binding sites are possible when the Hill coefficient is essentially unity.

The addition of sufficient malate to saturate the enzyme has a dramatic effect on the binding of NADH. The midpoint is slightly shifted to lower concentration. (At low ionic strength the midpoint is actually shifted to 0.1 μM NADH in the presence of L-malate.) The binding shows rather dramatic anticooperative character. As shown in Table I, the induced model requires a factor of about three increase in affinity while the asymmetry model requires about a fourfold strengthening of the binding. Both models predict about a factor of two weakening of the affinity for the second NADH binding site.

Although both alternative models predict similar changes in the NADH affinities as a result of malate binding, the two models are vastly different in their explanations of the mechanism of these changes in affinities. The induced model would imply that malate binding increases the communication between binding sites. The asymmetry model suggests that malate binding itself accentuates the differences in the binding sites and therefore results in a greater apparent anticooperativity in NADH binding.

It is interesting that malate binding in the presence of saturating NADH is also strongly anticooperative, showing the same Hill coefficient, 0.65 (Figure 7), as is observed for NADH binding in the presence of saturating malate (Figure 6). This reciprocal relationship is not required from the thermodynamics of the binding alone and must therefore reflect an important aspect of the mechanism of the anticooperative effects. Qualitatively, it would appear that the anticooperativity must be the result of the formation of ternary complexes. This implies that ternary complex formation induces changes in the interaction between binding sites. It is difficult to produce such an effect using a preexisting asymmetry model without interactions. For this reason we think the preexisting asymmetry model is less likely to be correct.

A number of models using induced differences are possible. A particularly simple example of such models supposes that a given subunit changes conformation only when malate *and* NADH are both bound. This is shown diagrammatically in Figure 8. Here NADH or malate binding alone is shown to be Michaelian with affinities of K_1 and K_1', respectively. When NADH binds to malate saturated enzyme, however, there is a conformation change and the equilibrium constant for the conformation change is K_T. In analogy to the original model proposed by Koshland et al. (1966) for tetrameric proteins, the interaction of the square and circle conformation is represented by the equilibrium constant K_{AB}. Thus the overall affinity constant for the first ligand is $K_1K_TK_{AB}$. The second NADH also causes conformation change in that subunit. The interaction of two square conformations is represented by K_{BB} and the overall

[2] Readers unfamiliar with the general case of ligand binding to a two site protein are directed to Edsall and Wyman (1958) for a complete discussion of this scheme.

affinity constant for the second NADH is therefore $K_1K_TK_{BB}/K_{AB}$. Using eq 3, the Hill coefficient for NADH binding to malate saturated enzyme is

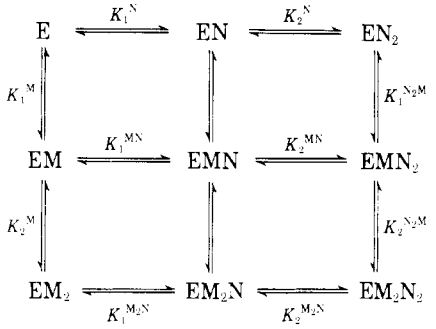
$$\eta_H = \frac{2}{[1 + (K_{AB}/(K)^{1/2})]}$$

The binding of malate to NADH-saturated enzyme follows a similar path. The first malate bound induces the same square conformation and is governed by the affinity constant $2K_1'K_TK_{AB}$. The second malate affinity constant is $K_1'K_TK_{BB}/K_{AB}$. The Hill coefficient becomes

$$\eta_H = \frac{2}{[1 + (K_{AB}/(K_{BB})^{1/2})]}$$

Thus the binding of NADH to malate-saturated enzyme and malate to NADH-saturated enzyme have the same anticooperativity for this model. Again it should be stressed that other models for the anticooperativity do not necessarily predict such an equivalence. The important feature of the model is that the only interactions of importance between the binding sites occur *only* in the ternary complexes. This generates the reciprocal relationship.

Considerable information concerning the binding of malate to the free enzyme can be inferred from our data although such binding was not determined directly because of the weak affinity of malate for the enzyme and the lack of any fluorescence change or other suitable probe of malate binding. This suggests the possibility that malate may not bind to the free enzyme, but only to enzyme sites containing NADH. However, if this were the case, the presence of malate should greatly stabilize NADH binding, thereby shifting the midpoint of the NADH binding curve to much lower concentration. In fact, the NADH midpoint is essentially the same (changing only by a factor of 10 rather than several orders of magnitude) in the presence or absence of malate. Therefore models must include L-malate binding to the free enzyme. This can be seen more clearly from the thermodynamic cycle



This shows the binding of NADH (N) and malate (M) to the enzyme E. Each binding step is represented by a different macroscopic phenomenological constant which describes the formation of all species of a given stoichiometry. Thus K_1^N describes the binding of NADH to free enzyme. The induced model would interpret K_1^N as $2K_1$ while the preexisting asymmetry model would interpret it as $(K_1 + K_1')$. Not all the constants shown are independent. In fact, eight independent constants are required to describe this situation. For example

$$K_1^N K_2^N K_1^{N_2M} K_2^{N_2M} = K_1^M K_2^M K_1^{M_2N} K_2^{M_2N} \quad (4)$$

That is, the formation constant of EM_2N_2 from free enzyme and free ligands must be the same if the path is to

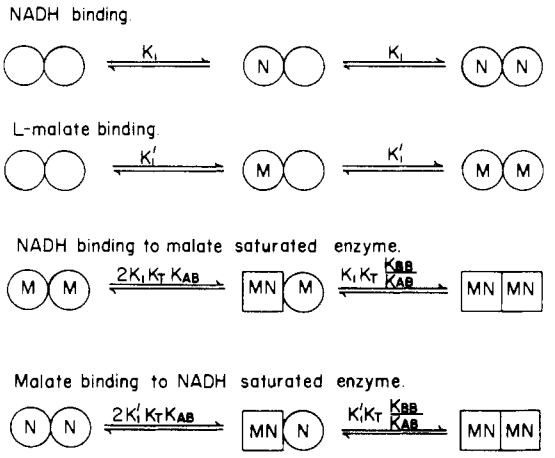


FIGURE 8: A ligand binding model consistent with the data presented in the Results section indicating the mechanistic significance of constants listed in Table I for the "induced model". The letter "N" represents NADH and the letter "M" represents L-malate. The adjoining circles represent apparently noninteracting subunits and the transformation to the square shape depicts the conformational change presumably inducing molecular asymmetry and anticooperative binding behavior.

first bind NADH and then malate or to first bind malate and then NADH. Thus we may infer the binding of malate to the free enzyme since

$$K_1^M K_2^M = \frac{K_1^N K_2^N}{K_1^{M_2N} K_2^{M_2N}} K_1^{N_2M} K_2^{N_2M} \quad (5)$$

The values on the right-hand side of eq 5 are all known since K_1K_2 for any ligand binding is simply the inverse of the midpoint concentration squared.

$$K_1^M K_2^M = \frac{(6.7 \times 10^{-7})^2}{(8.7 \times 10^{-7})^2}\left(\frac{1}{2.7 \times 10^{-3}}\right)^2 M^{-2} = 8 \times 10^4\ M^{-2}$$

The product $K_1^M K_2^M$ is related to the midpoint of the binding curve of malate to the free enzyme. The midpoint becomes

$$X_{0.5} = \left(\frac{1}{K_1^M K_2^M}\right)^{1/2} = 3.5 \times 10^{-3}\ M$$

Unfortunately, we have no accurate method of measuring $X_{0.5}$. Our induced model for the interaction of malate, NADH, and the enzyme predicts that such binding will be hyperbolic.

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The Role of Arginyl Residues in Directing Carboxymethylation of Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The selective carboxymethylation by iodoacetate of Cys-46 in the active center of horse liver alcohol dehydrogenase has been shown to be mediated by interaction of the anionic reagent with the arginyl residue(s) previously shown to be responsible for binding NADH (L. G. Lange, J. F. Riordan, and B. L. Vallee (1974), *Biochemistry 13,* 4361). Thus, sequential and reversible chemical modification of arginine with butanedione and of cysteine with *p*-mercuribenzoate demonstrate that the essential thiol groups are not affected by arginine modification. Importantly, the rate of incorporation of [^{14}C]iodoacetate into native horse liver alcohol dehydrogenase is ten times faster than that for the butanedione-modified enzyme. Moreover, as evidenced by peptide isolation, the radiolabel incorporated into the latter occurs at low levels in several different peptides as opposed to the single, strongly labeled CmCys-46 peptide obtained from the native enzyme. The demonstration that the arginyl residue(s) involved in coenzyme binding promotes enhanced reactivity of the active site thiol supports the general hypothesis that the spatial arrangement of structural features allowing expression of enzymatic function may also account for enhanced chemical reactivity of certain active site residues (B. L. Vallee and J. F. Riordan (1969), *Annu. Rev. Biochem. 38,* 733).

Selective carboxymethylation of horse liver alcohol dehydrogenase with iodoacetate has demonstrated the existence of a catalytically essential, active site cysteine residue (Li and Vallee, 1964) later shown to be Cys-46 (Jörnvall, 1970). Only iodoacetate reacts rapidly and specifically. Iodoacetamide, for example, reacts slowly and nonselectively (Li and Vallee, 1965). This observation suggests that a negatively charged carboxylate group might be instrumental in directing the reagent to the active site. Indeed, Reynolds and McKinley-McKee (1969) and Reynolds et al. (1970) have shown that prior to irreversible alkylation iodoacetate and enzyme form a reversible complex. NADH, ADPR,[1] and other anionic ligands such as chloride prevent this interaction competitively, consistent with the presence of a cationic site near Cys-46 which might bind both the coenzyme and the carboxylate group of iodoacetate. Further, the anion $Pt(CN)_4^{2-}$ employed in the preparation of isomorphous heavy atom derivatives of the enzyme for X-ray structure analysis inhibits horse liver alcohol dehydrogenase by competition with coenzyme (Gunnarsson et al., 1974) consistent with the existence of such a site.

Recently chemical modifications have shown that arginyl residues serve as NADH binding sites of the alcohol dehydrogenases from human and horse liver and from yeast (Lange et al., 1974). These results are entirely in accord with the results of X-ray structure analysis of the horse liver enzyme (Brändén et al., 1975). We have now employed se-

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[1] Abbreviations used are: ADPR, adenine diphosphoribose; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.