Malate Dehydrogenase. X. Fluorescence Microtitration Studies of D-Malate, Hydroxymalonate, Nicotinamide Dinucleotide, and Dihydronicotinamide–Adenine Dinucleotide Binding by Mitochondrial and Supernatant Porcine Heart Enzymes†

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ABSTRACT: Comparative binding studies of mitochondrial and supernatant malate dehydrogenases indicate that both enzymes evidently bind one NADH molecule per subunit. Both enzymes exhibit qualitatively similar behavior in binding NADH and hydroxymalonate less strongly with increasing pH. The NADH dissociation constant increases from about 0.4 to 8.2 μM in the mitochondrial and from about 0.2 to 3.6 μM in the supernatant enzyme as the pH is increased from 6.4 to 9.3. Similarly, the hydroxymalonate dissociation constant increases from 45 to about 620 μM in the mitochondrial and from about 520 to 4500 μM in the supernatant enzyme as the pH is increased from 6.3 to 9.3. The dissociation constant for NAD at pH 8.0 is 480 μM for the mitochondrial and 585 μM for the supernatant enzyme. NADH appears to be influenced, but not prevented from dissociation by the presence of hydroxymalonate in the ternary enzyme–NADH–hydroxymalonate complex. The equilibrium binding constants reported here agree well with those calculated from previously published kinetic data for the mitochondrial enzyme.

Although both the supernatant and mitochondrial malate dehydrogenases appear to have very similar, if not identical, subunits (Devenyi *et al.*, 1966) various recent publications suggest asymmetric behavior in the pig and beef heart enzymes. For example, supernatant beef heart enzyme has been reported (Cassman and Englard, 1966) to contain only one NADH[1] binding site per dimer. Recently, Glatthaar *et al.* (1972) have presented chemical evidence that this enzyme binds only one NAD per dimer in one of its crystal forms. Moreover, Tsernglou *et al.* (1971) have presented crystallographic evidence that a "small but definitive" conformational change, producing an asymmetrical dimer, occurs when one NAD molecule per dimer is bound in the enzyme crystal. Furthermore, initial rate kinetic data (Harada and Wolfe, 1968) have been interpreted in terms of functional interdependence between subunits in pig heart mitochondrial enzyme which might relate to asymmetrical structure–function relationships. These considerations as well as inconsistencies in the number of NADH binding sites which have been reported in the literature prompted the research to be described here.

Methods and Materials

All solution absorbance measurements were made with a Zeiss PMQ II spectrophotometer. Enzyme assays were carried out by adding the appropriate amount of enzyme in 10 μl to 3.0 ml of a solution at 25° which was 0.1 M in pH 10.0 glycine buffer, 0.001 M in NAD, and 0.01 M in malate. The rate of optical density change was measured with the use of a Hilger-Gilford recording spectrophotometer and a cuvet having a 1.0-cm light path.

Reagents. C. F. Boehringer and Soehne, G.m.b.H. (Grade II) NADH and NAD were purified by column chromatography on the day they were to be used. The NADH, eluted from a 3 × 15 cm DEAE-cellulose column with the use of a 300-ml 0–1.0 M $NaHCO_3$ gradient, had an optical absorbance ratio, 340 mμ/260 mμ, of 0.45. After washing with 400 ml of water, NAD was eluted from a 2 × 12 cm column of Dowex-1 with the use of 15 mM HCl. NADH concentration was determined by absorbance measurement at 340 mμ and NAD concentration by absorbance measurement at 260 mμ assuming ϵ_{340} and ϵ_{260} to be 6.22×10^3 and 18×10^3 for NADH and NAD, respectively. L-Malic acid was obtained from Koch-Light and hydroxymalonate (tartronate) from Ralph N. Emanuel Research Chemicals. Tris was Sigma primary standard and all Tris buffers were 0.05 M with respect to acetate.

Supernatant malate dehydrogenase was purchased from Seravac Laboratories. This enzyme had a specific activity of 80 IU/mg when assayed as described above and assuming $A_{280}^{1\%}$ 9.0 (Gerding and Wolfe, 1969) for the pure protein. The mitochondrial enzyme, prepared from pig heart acetone powder by modifications of the methods of Wolfe and Neilands (1956) and Thorne (1962), had a specific activity of 220 IU/mg assuming $A_{280}^{1\%}$ 2.7[2] for the pure enzyme. Little or no tryptophan fluorescence attested to the high purity of this preparation.

Titration. The natural fluorescence of NADH is enhanced by binding to both of the enzymes. Further fluorescence enhancement occurs when either hydroxymalonate[3] or D-malate

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[1] Abbreviations used are: NAD, nicotinamide–adenine dinucleotide; NADH, dihydronicotinamide–adenine dinucleotide; k_{oas}, oxalacetate Michaelis constant; k_m, malate Michaelis constant; K_{NAD}, NAD dissociation constant; K_{NADH}, NADH dissociation constant; and K_{NADH}^{app}, the apparent NADH dissociation constant.

[2] Unpublished value, R. G. Wolfe, in good agreement with $A_{280}^{1\%}$ 2.8 (Thorne and Kaplan, 1963).

[3] J. Shore, personal communication.

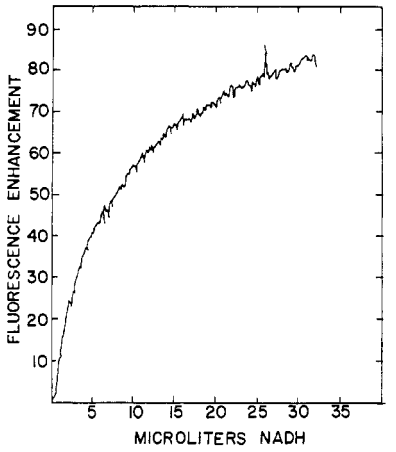

FIGURE 1: A tracing of the recorder graph as an example of the sensitivity of the fluorescence NADH binding titration technique. The ordinate represents the fluorescence difference ((enzyme-NADH)-(NADH)), uncorrected for dilution and quenching, in arbitrary units. The abscissa represents the number of microliters of 1.85 mM NADH added to a 3.0-ml volume of a solution containing 39 μg (2.2 nmoles of enzyme sites) of *S*-MDH in pH 8.0 Tris buffer which was 0.05 M with respect to acetate.

(Theorell and Langen, 1960) is bound by the enzyme–NADH binary complex. Because pure NAD does not fluoresce, the displacement of NADH from the enzyme–NADH binary complex by relatively high NAD concentrations causes a decrease in the fluorescence intensity. These changes in fluorescence intensity, which are closely proportional to changes in the quantity of ligand bound, were exploited in measuring the various dissociation constants by titration with the use of a double-beam fluorescence microtitration apparatus which was designed and constructed in this laboratory.[4]

The variable ligand, usually NADH, was added to both cuvets at a constant rate. The suitably amplified difference fluorescence signal, after conversion to digital form, was corrected for dilution, quenching, and absorbance change errors with a suitable computer program. The corrected data were then analyzed, assuming simple binding behavior, with a computer to determine the best theoretical fit values for the dissociation constant and the binding site concentration. The computed binding site concentration was then compared with the enzyme concentration as determined from specific activity and protein absorbance measurements. The pH was measured after completion of the titration experiment.

The NADH dissociation constants were determined at three pH values (pH 6.4, 8.0, and 9.3) by adding NADH from both syringes to the two cuvets, one of which contained enzyme at a concentration of about 0.1–1.0 μM (in NADH binding sites).

The NAD dissociation constants were determined by titrating NADH into both cuvets, each of which contained NAD and only one of which contained enzyme. It was assumed that simple competition occurred between NAD and NADH for the coenzyme binding site. The apparent dissociation constant for NADH was determined at pH 8.0 at each of a series of NAD concentrations. From these apparent values the NAD dissociation constants were calculated as shown in the Results section.

The dissociation constants for D-malate and hydroxymalonate were determined by titrating D-malate at 0.095 M or hydroxymalonate at 0.5 M into the two cuvets, each of which contained 30 μM NADH and one of which contained enzyme. The data were treated in the same manner as that described for the NADH titration above.

The influence of hydroxymalonate on NADH binding was studied by titrating NADH into both cuvets containing 0.01 M hydroxymalonate and one of which contained enzyme.

[4] By J. J. Holbrook.

TABLE I: Summary of Binding Site Data.

	Concentration of NADH Binding Site Concentration (μM)[a]	Subunit Concentration Based on Enzyme Concentration Measurement (μM)	NADH Binding Sites per Subunit
	Mitochondrial Malate Dehydrogenase		
1	7.29	8.5[c]	0.86
2	7.33	8.5	0.86
3	3.95[b]	4.2	0.94
	Supernatant Malate Dehydrogenase		
4	5.54	5.07[d]	1.09
5	1.02	1.01	1.0

[a] Except for one experiment (line 4) with supernatant malate dehydrogenase, which was titrated at pH 8.0, all titrations for both enzymes were carried out at pH 6.4 in 0.05 M Tris acetate buffer at 25°. [b] This binding site titration was carried out in the presence of 0.01 M hydroxymalonate in order to strengthen NADH binding through ternary complex formation. [c] The concentration of mitochondrial enzyme was determined assuming a subunit weight of 35,000 and $A_{280}^{1\%}$ 2.7 (R. G. Wolfe, unpublished) for the pure protein. [d] Supernatant enzyme concentration was calculated by assuming a subunit molecular weight of 37,000 and $A_{280}^{1\%}$ 9.0 (Gerding and Wolfe, 1969) for the pure enzyme. Also, the concentration, calculated assuming specific activity of the pure supernatant enzyme to be 110 IU/mg of pure protein, was in good agreement with concentration calculated with absorbance measurements. Correction was made for the impurities by use of specific activity ratios (110/80) in calculating subunit concentration by this method.

Results and Discussion

Figure 1 shows an example titration graph illustrating the very high sensitivity of the method. A 39-μg quantity of the supernatant enzyme was titrated with 1.85 mM NADH in a 3.0-ml volume in this experiment. Reproducible dissociation constant values may be obtained with half this quantity of enzyme.

The calculated values for the number of NADH binding sites for both the mitochondrial and supernatant enzymes are summarized in Table I. These data are consistent with the occurrence of one coenzyme binding site per subunit in each form of the enzyme. Linear Hill plots attest to the occurrence of uncomplicated coenzyme binding within the limits of detectability by this method.

The supernatant beef heart enzyme has been reported to have but one NADH binding site per 52,000 daltons by Cassman and Englard (1966) (see also Wolfenstein *et al.*, 1969). The number of binding sites in the mitochondrial en-

TABLE II: Summary of NADH Dissociation Constants.

pH[a]	NADH Dissociation Constant (μM) for Mitochondrial Malate Dehydrogenase	NADH Dissociation Constant (μM) for Supernatant Malate Dehydrogenase
9.3	8.2[c]	3.6
9.3	4.0[c] (2.9)[b]	3.6
8.0	2.16	1.4
8.0	2.0 (0.52)	0.80
6.4	0.52	0.17
6.4	0.48 (0.086)	
6.4	0.37 (0.05)	

[a] All titrations were carried out at 25° in Tris buffer which was 0.05 M with respect to acetate. [b] Values of the dissociation constants in parentheses are apparent NADH dissociation constants in the presence of 0.01 M hydroxymalonate. [c] Mitochondrial malate dehydrogenase dissociation constants were variable (4–8 μM), possibly because of enzyme instability at the higher pH.

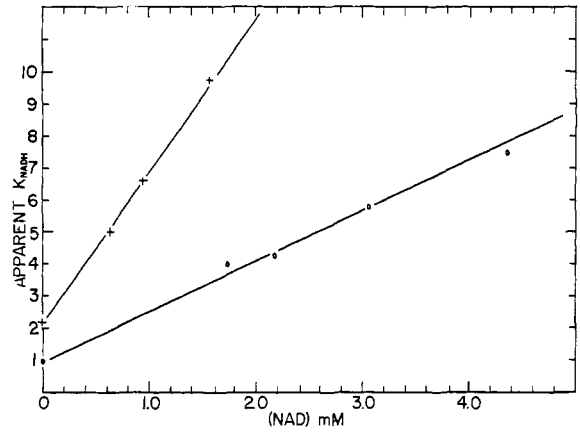

FIGURE 2: Experimental values for the apparent NADH dissociation constant are plotted (crosses for mitrochondrial and circles for the supernatant enzyme) as a function of the NAD concentration at which each value was determined. The titrations were all carried out at pH 8.0 in Tris buffer which was 0.05 M with respect to acetate and at 25°. The linear mathematical equation relevant to this plot is presented in the text.

zyme has been variously reported to be 4.4 sites per 40,000 daltons (Theorell and Langen, 1960), one per 34,000 (Thorne and Kaplan, 1963), one per 40,000 (Pfleiderer, 1960), and 2 per 85,000 (Pfleiderer and Aurricchio, 1964). With the exception of the first two citations above, all of these values support the occurrence of one NADH binding site per subunit in agreement with the results reported in this paper.

As shown in Table II, both mitochondrial and supernatant malate dehydrogenase bind NADH less tightly with increasing pH although the supernatant enzyme consistently binds NADH more tightly than does the mitochondrial enzyme under the same conditions. Theorell and Langen (1960) have reported pig heart mitochondrial malate dehydrogenase to have a dissociation constant of 1.0 μM at pH 7.15 in phosphate buffer at 23.5°. This value falls in the expected range by interpolation of the values presented in Table II although different buffers were used in the two studies. Anderton and Rabin (1970) report the NADH dissociation constant to be 10 μM in phosphate buffer of pH 7.8 and an ionic strength of 0.2. Apparently the dissociation constant increases with increasing ionic strength.

The NAD dissociation constant is related to the NADH dissociation constant (K_{NADH}) in the presence of NAD by the following expression: $K_{NADH}^{app} = K_{NADH}(1 + (NAD)/K_{NAD})$. Experimental apparent NADH dissociation constants for both forms of the enzyme are plotted against the NAD concentration in Figure 2. It is evident that NAD is more tightly bound by the mitochondrial form of the enzyme. K_{NAD} is 480 ± 100 μM for the mitochondrial enzyme and 585 ± 100 μM for the supernatant enzyme under these experimental conditions. The NAD dissociation constant for mitochondrial malate dehydrogenase has been reported by Theorell and Langen (1960) to be 280 μM at pH 7.15 and 23.5°. Our measurements at pH 8.0 in Tris acetate at 25° give a value, 480 μM, which, for lack of pH dependence data, cannot be directly compared with Theorell and Langen's result. Our result is in agreement with the value (400 μM) reported by Anderton and Rabin (1970).

Because of the relatively large dissociation constants for hydroxymalonate and D-malate, it was not possible to evaluate the number of binding sites for these ligands.

Table III summarizes the dissociation constant values for hydroxymalonate, comparing the supernatant and mitochondrial enzymes at three pH values. It is evident that the mitochondrial enzyme binds hydroxymalonate more tightly than does the supernatant enzyme and that both forms of the enzyme bind the hydroxymalonate less tightly with increasing pH. Comparison of this pig heart enzyme with that from beef heart in the work of Cassman and Englard (1966) indicates that the latter has a hydroxymalonate dissociation constant of 1500 μM at pH 8.2 whereas the nearest comparable value reported in this paper is about 500 μM at pH 8.0. Apparently, the hydroxymalonate dissociation constant is slightly larger in the beef heart enzyme although the pig heart enzyme value seems to increase sharply with increasing pH above pH 8.0 as shown in Table III.

It is interesting that the trend toward tighter ligand binding as the pH is lowered applies to both enzymes and to NADH as well as hydroxymalonate. Also, although D-malate resembles the structure of the natural substrate more closely than does hydroxymalonate, the latter is apparently bound 50 times more strongly in the enzyme–NADH binary complex of the mitochondrial enzyme.

TABLE III: Summary of Hydroxymalonate Dissociation Constants.

pH[a]	Mitochondrial Enzyme K_{HM} (μM)	Supernatant Enzyme K_{HM} (μM)
6.3		520
6.4	45	
6.4	69	
8.0	103	670
8.0	140	400
9.3	620	4500

[a] All buffers were Tris which was 0.05 M with respect to acetate and the temperature was 25°.

The numbers in parentheses in Table II represent the apparent NADH dissociation constants measured in the presence of 0.01 M hydroxymalonate. These values are far greater than predicted by $K_{NADH}^{app} = K_{NADH}/(1 + (\text{hydroxymalonate})/k_{HM})$ indicating that NADH can dissociate from the ternary complex, although it is influenced by hydroxymalonate binding.

The dissociation constant has been evaluated for D-malate in the case of the mitochondrial enzyme only. The value of the dissociation constant was 2.3 mM at pH 6.4 and 25°. Very different experimental conditions employed by Theorell and Langen (1960) probably account for the considerable difference of the value obtained by them (14.9 mM) for this ligand. Their larger value might be accounted for by less tight binding at higher pH values in a manner of behavior similar to that observed for hydroxymalonate. Cassman and Englard (1966) reported the beef heart enzyme to have a dissociation constant for D-malate of 500 μM at pH 8.2 in triethanolamine buffer. The pig and beef heart enzymes appear to differ significantly in this property.

Steady-state kinetic data (Raval and Wolfe, 1962) as well as equilibrium isotope rate data (Silverstein and Sulebele, 1969) indicate that the mitochondrial enzyme has a compulsory order mechanism in which coenzyme is bound first and coenzyme-product is released last at pH 8.0. Assuming this mechanism, it is possible to calculate the rate constants relevant to the two coenzyme dissociation constants from the previously published steady-state data (Raval and Wolfe, 1962). The kinetic data yield values of 1.0 mM and 5.2 μM for K_{NAD} and K_{NADH}, respectively. These values compare well with 0.48 mM and 4.7 μM equilibrium measurements (average of pH 8.0 values, Table II) reported here.

The data reported here on the occurrence of two NADH binding sites are in agreement with most of the published work on this subject. Complications arise in explaining apparent asymmetric properties of these enzymes in the face of apparently linear Hill plots of the binding data for NADH. Apparent asymmetry in kinetic data (Harada and Wolfe, 1968) may somehow depend on the execution of the complete catalyzed reaction and remain unexpressed in the partial reactions reported here. The perplexing asymmetry reported by Glatthaar *et al.* (1972) and Tserglou *et al.* (1971) is not explainable in this way. Possibly the asymmetric coenzyme binding in this case is related to the use of NAD rather than NADH as the ligand or to some unique properties of the enzyme crystal structure.

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