Interaction of Reduced Nicotinamide Adenine Dinucleotide with Beef Heart s-Malate Dehydrogenase†

Ruth Koren and Gordon G. Hammes*

ABSTRACT: The interaction of NADH with s-malate dehydrogenase isolated from beef heart was studied in 20 m*M* potassium phosphate (pH 6.9)–1 m*M* EDTA, with forced dialysis, fluorescence, and temperature-jump techniques. Measurements of the change in fluorescence of NADH when it is titrated with enzyme indicate NADH bound to monomeric and dimeric enzyme have different fluorescence yields. These data and the results of direct binding studies can be explained in terms of a model in which the NADH binding sites on dimeric enzyme are equivalent or nearly equivalent, and NADH binding to monomeric enzyme occurs with an affinity very similar to that of the dimer. However, the fluorescence enhancement of NADH on binding to the enzyme is different for the monomer and for each of the two dimer sites.

The enzyme s-malate dehydrogenase undergoes a monomer–dimer reaction with an association constant of approximately 10^7 M^{-1} (Cassman and King, 1972). The binding of NADH to the enzyme is accompanied by an enhancement of the fluorescence of NADH and a quenching of the protein fluorescence. Fluorescence titrations of the enzyme with NADH have been used to characterize the binding process (Cassman and King, 1972). If the fluorescence changes are assumed to be linear functions of the number of ligand binding sites occupied, the binding isotherms calculated from the titrations exhibit positive cooperativity. However, thus far is has not been possible to construct a model which quantitatively describes the binding isotherms over a wide range of protein and NADH concentrations.

In this work, the interaction between NADH and s-malate dehydrogenase has been studied using direct binding measurements and fluorescence titrations. The results obtained can be explained by a model in which NADH binds to both monomeric and dimeric enzyme. The binding sites of the dimer are nearly equivalent or equivalent, and the affinity for the monomer site is very similar to that for the dimer sites. However, the fluorescence enhancement of NADH on binding to monomer and the two dimer sites has a different value for each of the three types of sites.

Experimental Section

Materials. The NADH was purchased from Sigma (grade III disodium salt). A single ultraviolet and fluorescent spot was observed when the NADH was chromatographed on DEAE paper in aqueous 0.1 *M* NH_4HCO_3 (Silverstein, 1970). Solutions of NADH were prepared immediately before use, and their concentration was determined by absorbance measurements at 340 nm using an extinction coefficient of 6.22 10^3 M^{-1} cm^{-1} (Horecker and Kornberg, 1948). Radioactive [^{14}C]NAD^+ was purchased from Amersham/Searle (100–250 Ci/mol). The NAD^+ was converted to NADH enzymatically with alcohol dehydrogenase (Klingenberg, 1963) in 0.1 *M* Tris buffer (pH 8.8) and 0.5 *M* ethanol, and purified on a DEAE-cellulose column (Silverstein, 1965). In a control experiment the NADH prepared in this manner was found to be at least 96% pure by enzymatic assay with lactic dehydrogenase (Klingenberg, 1963).

The s-malate dehydrogenase was purified from fresh beef heart as previously described (Englard and Breiger, 1962; Guha et al., 1968). The specific activity of the enzyme preparations used in this work was between 450 and 500 units/mg. On acrylamide gel electrophoresis in 0.2 *M* glycine–0.025 *M* Tris (pH 8.3), the enzyme moved in two close bands, one much fainter than the other. A similar result has been obtained for the pig heart enzyme (Gerding and Wolfe, 1968).

All other chemicals used were the best available commercial grades, and solutions were made with distilled deionized water.

Analytical Methods. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Enzyme concentrations were calculated in terms of monomer units of molecular weight 40,000. The enzyme was assayed spectrophotometrically at 30° with a Cary 14 recording spectrophotometer (Guha et al., 1968). The enzyme activity did not change significantly during the course of the experiments described below.

Binding Measurements. The binding of NADH to s-malate dehydrogenase was studied using the forced dialysis method with radioactive NADH (Cantley and Hammes, 1973). Diaflo-PM-10 membranes were used in a Metalloglas ultrafiltration apparatus after first soaking the membranes in water. All binding measurements were carried out in 20 m*M* potassium phosphate (pH 6.9)–1 m*M* EDTA, with an enzyme concentration of 1.84 or 4.35 μM and varying NADH concentrations (0.5–28 μM). The total ligand concentration was determined by measuring the radioactivity of a 10-μl aliquot in Bray's scintillation fluid (Bray, 1960) with a Beckman LS255 liquid scintillation counter. A 0.25–0.30-ml aliquot of solution was placed in each cell compartment and a pressure of approximately 10 psi nitrogen was applied. The first 10 μl of solution forced through the membrane was discarded since the NADH is diluted by the water present in the membranes. The second 10 μl

† From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received October 18, 1974.* This work was supported by a grant from the National Institutes of Health (GM 13292).

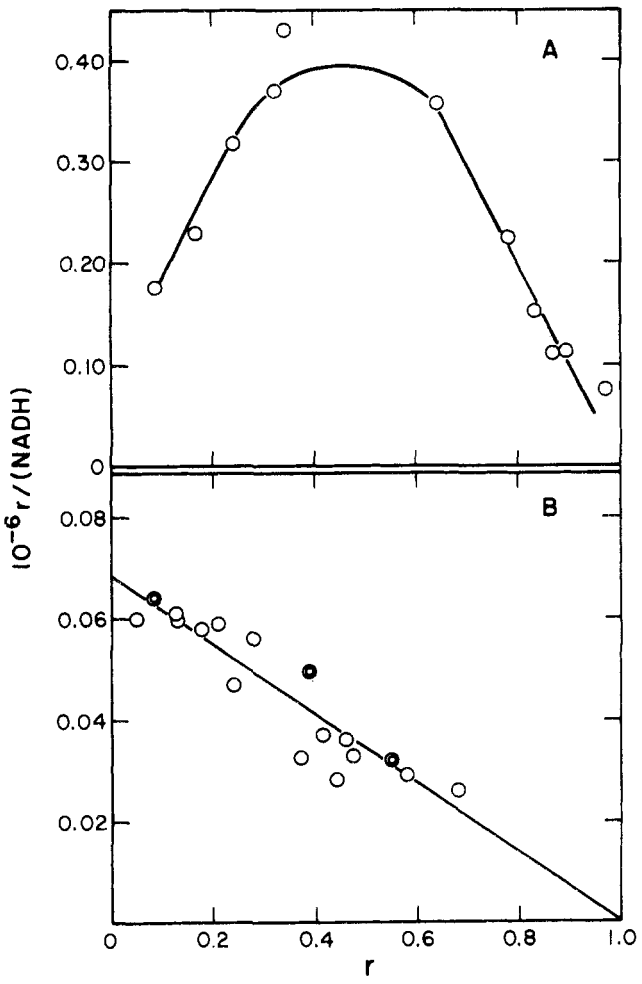

FIGURE 1: Plots of the moles of NADH bound per mole of enzyme (molecular weight 40,000) divided by the free NADH concentration, $r/(NADH)$, vs. r at an enzyme concentration of 4.35 μM in 20 mM potassium phosphate (pH 6.9)–1 mM EDTA at 24°. (A) Data obtained from a fluorescence titration analyzed according to the method of Cassman and King (1972). (B) Data obtained from direct binding measurements using forced dialysis.

forced through the membrane was used to measure the concentration of free NADH by radioactive assay. The fact that the concentration of the second sample is not diluted was determined by control experiments with NADH in the absence of enzyme. In some cases, a third 10-μl aliquot also was analyzed and within the experimental uncertainties had a radioactivity identical with that of the second 10-μl aliquot. The enzyme also was found to be retained by the membrane in control experiments.

Fluorescence Titrations. Fluorescence measurements were carried out at 20 or 24° with a Perkin-Elmer MPF-3 fluorescence spectrophotometer. All fluorescent measurements on NADH were made at an excitation wavelength of 340 nm and an emission wavelength of 410 nm. The excitation slit was 6 nm and the emission slit was 8 nm.

The fluorescence intensity of a 24 μM NADH solution in 20 mM phosphate (pH 6.9)–1 mM EDTA was determined at varying enzyme concentrations. This was accomplished by first measuring the fluorescence of 24 μM NADH in the presence of 2 μM enzyme. A 0.4-ml aliquot was then removed from the 2 ml in the 3-ml fluorescence cell and was replaced with the same volume of a 24 μM NADH stock solution; the fluorescence of the resulting solution was then measured. This procedure was repeated until no change in fluorescence intensity could be detected after dilution. The fluorescence intensity of the NADH stock solution was measured separately in an identical cell and was found to be identical with the fluorescence intensity of the cell after the final dilution.

Fluorescent titrations of enzyme with NADH were carried out under identical conditions with those described above. The titrations were performed in a 3-ml fluorescence cell containing 2 ml of buffer solution and a known enzyme concentration. Concentrated NADH was added to the cell in 2–30-μl aliquots, and the fluorescence intensity was measured. To correct for dilution and inner-filter effects, the same titration was carried out in an identical cell containing only buffer solution without protein. No more than five dilutions were made on a single solution, and all measurements were done at least in duplicate. The range of enzyme concentrations used was 1.12–7.98 μM and that for NADH was 0.1–36 μM.

Temperature-Jump Experiments. Relaxation experiments were performed with a temperature-jump apparatus utilizing fluorescence detection (del Rosario, 1970). The temperature jump applied was 7.7° to a final temperature of either 15.7 or 22.7°. The excitation wavelength was 340 nm and the emitted light was passed through a filter (LS-0-51, Corning) to minimize the contributions of scattered light and the Raman peak. All solutions contained 16 mM potassium phosphate, 0.8 mM EDTA, and 0.2 M KNO_3. The enzyme concentration varied from 1 to 3 μM and the NADH concentration from 2.5 to 20 μM. Photographs of relaxation processes were taken, and the relaxation times were determined from plots of the logarithm of the signal amplitude vs. time.

Results and Treatment of Data

A complete thermodynamic description of the interaction of NADH with *s*-malate dehydrogenase requires four independent chemical equations. These equations must take into account the facts that the enzyme exists in both monomer and dimer forms and that each monomer unit has a single NADH binding site (Cassman and King, 1972). One set of four independent chemical reactions is given in eq 1–4. In

$$E + E \rightleftharpoons E_2 \qquad K_D = (E_2)/(E)^2 \qquad (1)$$

$$E + S \rightleftharpoons ES \qquad K_{ES} = (ES)/(E)(S) \qquad (2)$$

$$E_2 + S \rightleftharpoons E_2S \qquad K_{E_2S} = (E_2S)/(E_2)(S) \qquad (3)$$

$$E_2S + S \rightleftharpoons E_2S_2 \qquad K_{E_2S_2} = (E_2S_2)/(E_2S)(S) \qquad (4)$$

these equations E denotes monomeric enzyme, E_2 dimeric enzyme, and S is NADH. All other chemical equilibria which occur can be written in terms of these four reactions.

The NADH–enzyme binding constants have been determined by Cassman and King (1972) by fluorescence titrations, assuming the fluorescence enhancements accompanying NADH binding to the enzyme are identical for monomer and both dimer binding sites. In Figure 1A, a plot of the moles of NADH bound per mole of enzyme of molecular weight 40,000, r, divided by the concentration of free NADH is plotted vs. r; these data have been derived from a fluorescence titration analyzed according to the method of Cassman and King (1972). This plot is in reasonable agreement with those presented by Cassman and King (1972) and suggests positive cooperativity occurs in the binding process. However, in Figure 1B, a similar plot is shown which is derived from direct binding measurements under the same experimental conditions (4.35 μM enzyme–20

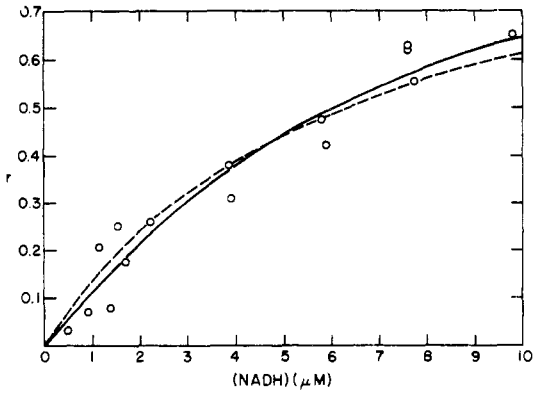

FIGURE 2: A plot of the moles of NADH bound per mole of enzyme (molecular weight 40,000), r, vs. the concentration of free NADH as determined by forced dialysis in 20 mM potassium phosphate (pH 6.9)–1 mM EDTA at 20°. The enzyme concentration was 1.84 μM. The solid line has been calculated with eq 6 and $K_D = 8.5 \times 10^6\ M^{-1}$, $K_{ES} = 0.21 \times 10^6\ M^{-1}$, and $K_{E_2S} = 0.18 \times 10^6\ M^{-1}$. $K_{E_2S_2} = 0.14 \times 10^6\ M^{-1}$, while the dashed line has been calculated both with eq 6 and $K_D = 10.1 \times 10^6\ M^{-1}$, $K_{ES} = 0.22 \times 10^6\ M^{-1}$, $K_{E_2S}' = 0.15 \times 10^6\ M^{-1}$ ($=K_{E_2S}/2 = 2K_{E_2S_2}$) and with eq 5 and $K = 0.16\ \mu M^{-1}$.

mM phosphate, pH 6.9, 24°). No cooperativity is evident in the binding process, and if only a single class of binding sites is assumed to be present so that r is given by eq 5, a

$$r = K(\text{NADH})/[1 + K(\text{NADH})] \qquad (5)$$

binding constant, K, of 0.07 μM^{-1} is obtained from a least-squares analysis of the data. The difference between the plots obtained from the fluorescence titration and the direct binding studies suggests that the assumption of identical fluorescence enhancements for all of the ligand binding sites is not valid for this system.

Since at 4.35 μM enzyme the enzyme is essentially all dimer (Cassman and King, 1972), little information can be obtained from the data in Figure 1 about the binding characteristics of the monomer. Therefore, direct binding measurements also were carried out at a lower enzyme concentration, 1.84 μM (20°). The results obtained are presented in Figure 2 as a plot of r vs. (NADH). Again no cooperativity is apparent in the binding process. If these data are analyzed assuming a single type of binding site (eq 5), a ligand binding constant of 0.16 μM^{-1} is obtained. The dashed line in Figure 2 has been calculated with eq 5 assuming this constant. These data also can be analyzed according to the more general binding scheme of eq 1–4. In this case r can be written as in eq 6. In principle, the data can be fit directly to eq 6 by a nonlinear least-squares analysis and the four equilibrium constants can be determined since (S) is known and (E) can be calculated from eq 7, which has been derived from mass balance, and eq 1–4. In eq 7 (E_0) is the total enzyme concentration. However, in practice it was found that the data are insufficient to determine uniquely all four constants.

$$r = \frac{(ES) + (E_2S) + 2(E_2S_2)}{(E) + (ES) + 2(E_2) + 2(E_2S) + 2(E_2S_2)} = \frac{K_{ES}(S) + K_D(E)[K_{E_2S}(S) + 2K_{E_2S}K_{E_2S_2}(S)^2]}{1 + K_{ES}(S) + 2K_D(E)[1 + K_{E_2S}(S) + K_{E_2S}K_{E_2S_2}(S)^2]} \qquad (6)$$

$$(E) = \{-1 + \sqrt{1 + 2(E_0)\alpha/[1 + K_{ES}(S)]}\}/\alpha$$
$$\alpha = \frac{4K_D[1 + K_{E_2S}(S) + K_{E_2S}K_{E_2S_2}(S)^2]}{1 + K_{ES}(S)} \qquad (7)$$

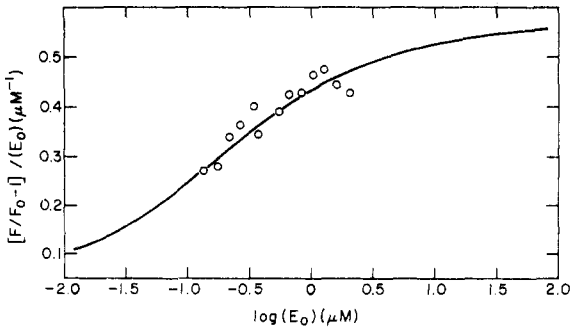

FIGURE 3: A plot of the fluorescence enhancement, $(F/F_0 - 1)$, divided by the total enzyme concentration (E_0), vs. the logarithm of the total enzyme concentration. The titration was carried out in 24 μM NADH–20 mM potassium phosphate (pH 6.9)–1 mM EDTA at 20°. The solid line was calculated with eq 8 and $K_D' = 4.5 \times 10^6\ M^{-1}$, $\lambda_{ES} = 2.5$, and $\lambda_{E_2S_2} = 14.9$.

The fluorescence titration of a constant NADH concentration with enzyme can be used to provide an additional relationship which reduces the number of unknown parameters in eq 6 to three. The NADH concentration in these experiments was made as high as possible (consistent with obtaining reliable fluorescence data) and the enzyme concentration was always much lower than the NADH concentration. Therefore, if the binding of NADH to the enzyme is sufficiently tight the assumption can be made that the only enzyme species present in solution are ES and E_2S_2. The validity of this assumption can be checked later when all of the equilibrium constants for the system are known: as it turns out at least 80–90% of the enzyme is saturated with substrate under the experimental conditions employed. The fluorescence intensity of the sample, F, relative to that of NADH in the absence of enzyme, F_0, is given by eq 8. In this equation (S_0) is the total concentration of (NADH), λ_{ES} is the fluorescence enhancement of NADH upon binding to monomer, and $\lambda_{E_2S_2}$ is the fluorescence enhancement of NADH (per NADH bound) upon binding to the dimer. A plot of $(F/F_0 - 1)/(E_0)$ vs. log (E_0) at an NADH concentration of 24 μM is presented in Figure 3. The shape of this curve is determined entirely by a single thermodynamic constant $K_D' = (E_2S_2)/(ES)^2$. The unknown fluorescent enhancements serve only to define the ordinate scale. The data in Figure 3 were fit to eq 8 by a nonlinear least-squares analysis, and the values of the parameters obtained were $K_D' = 4.5 \times 10^6\ M^{-1}$, $\lambda_{ES} = 2.5$, and $\lambda_{E_2S_2} = 14.9$. The curve in Figure 3 has been calculated with these parameters and eq 8. Because the enzyme is only 80–90% saturated, the values of the fluorescent enhancements and K_D' are somewhat in error. However, the error made is not great enough to significantly alter the subsequent analysis and the conclusions reached.

$$(F/F_0 - 1)/(E_0) = [(\lambda_{ES} - 1)(ES)/(E_0) + 2(\lambda_{E_2S_2} - 1)(E_2S_2)/(E_0)]/(S_0) \qquad (8)$$

Since $K_D' = K_D K_{E_2S} K_{E_2S_2}/(K_{ES}^2)$, one of the parameters in eq 6 can now be eliminated. The data in Figure 2 were fit to eq 5, modified by the insertion of K_D', with a nonlinear least-squares analysis. The four equilibrium constants obtained from this analysis are $K_D = 8.5 \times 10^6\ M^{-1}$, $K_{ES} = 0.21 \times 10^6\ M^{-1}$, $K_{E_2S} = 0.18 \times 10^6\ M^{-1}$, and $K_{E_2S_2} = 0.14 \times 10^6\ M^{-1}$. The solid line in Figure 2 has been cal-

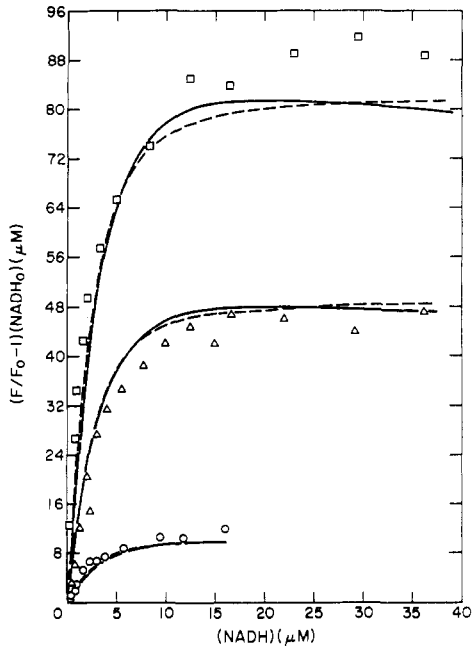

FIGURE 4: A plot of the product of the fluorescence enhancement ($F/F_0 - 1$) and the total NADH concentration, ($NADH_0$), vs. the concentration of free NADH. The titrations were carried out in 20 mM potassium phosphate (pH 6.9)-1 mM EDTA at 20° with enzyme concentrations of 1.12 μM (○), 4.84 μM (△), or 7.98 μM (□). The solid lines were calculated with eq 9 and $K_D = 8.5 \times 10^6\ M^{-1}$, $K_{ES} = 0.21 \times 10^6\ M^{-1}$, $K_{E_2S} = 0.18 \times 10^6\ M^{-1}$, $K_{E_2S_2} = 0.14 \times 10^6\ M^{-1}$, $\lambda_{ES} = 3$, $\lambda_{E_2S} = 37$, and $\lambda_{E_2S_2} = 11$, while the dashed line was calculated with eq 9, and $K_D = 10.1 \times 10^6\ M^{-1}$, $K_{ES} = 0.22 \times 10^6\ M^{-1}$, $K_{E_2S}' = 0.15 \times 10^6\ M^{-1}$ ($=K_{E_2S}/2 = 2K_{E_2S_2}$), $\lambda_{ES} = 1.02$, $\lambda_{E_2S} = 24.6$, and $\lambda_{E_2S_2} = 12$.

culated with these parameters and eq 6. An initial value of $10^7\ M^{-1}$ was assumed for K_D in the fitting procedure, and least-squares deviations between the calculated and observed data points were not significantly altered over a wide range of values of K_D. The initial value of $10^7\ M^{-1}$ was estimated from ultracentrifuge data (Cassman and King, 1972) so that this constant is semiquantitatively fixed by an independent experiment. The values of the binding constants for the dimer are sufficiently similar to suggest that two equivalent sites may exist. This assumption is supported by the results presented earlier in Figure 1 at 4.35 μM enzyme, a concentration at which the enzyme is essentially dimeric both in the presence and absence of NADH. Therefore, the data in Figure 1 were fit to eq 6 with the additional assumption $K_{E_2S} = 2K_{E_2S_2}'$ and $K_{E_2S_2} = K_{E_2S_2}'/2$ where $K_{E_2S_2}'$ is the intrinsic binding constant for the NADH–dimer interaction. (The known value of K_D' was used to reduce the number of parameters to two.) The equilibrium constants obtained from this analysis are $K_D = 10.1 \times 10^6\ M^{-1}$, $K_{ES} = 0.22 \times 10^6\ M^{-1}$, and $K_{E_2S_2}' = 0.15 \times 10^6\ M^{-1}$. The dashed curve in Figure 1, which is identical with the curve calculated earlier with eq 5, has been calculated with these constants and eq 6. Again this analysis determines K_D very imprecisely. The direct binding data obtained at a higher enzyme concentration and slightly higher temperature (24 vs. 20°) shown in Figure 1 suggest a smaller value of $K_{E_2S_2}'$ ($0.07\ \mu M^{-1}$), but the difference is within the experimental uncertainty and the expected decrease in the binding affinity at the somewhat higher temperature. Similar titrations to those performed by Cassman and King (1972) have been carried out in this work in order to determine the fluorescence enhancements for the various enzyme species. The results obtained at three different enzyme concentrations, 1.12, 4.84, and 7.98 μM, are shown in Figure 4. In this figure, the product of the fluorescent enhancement and the total NADH concentration $(F/F_0 - 1)(NADH_0)$ is plotted vs. the free NADH concentration calculated with the equilibrium binding constants given above (assuming nonequivalent sites). In analogy with eq 8, the fluorescent enhancement can be written as

$$(F/F_0 - 1)(S_0) = (\lambda_{ES} - 1)(ES) + (\lambda_{E_2S} - 1)(ES_2) + 2(\lambda_{E_2S_2} - 1)(E_2S_2) \quad (9)$$

Since the binding constants have been determined independently, only the fluorescent enhancements are unknown, and the three sets of data in Figure 4 have been fit by a nonlinear least-squares analysis to obtain the fluorescent enhancements. The values of the enhancements obtained using the constants assuming nonequivalent dimer sites are $\lambda_{ES} = 3$, $\lambda_{E_2S} = 37$, and $\lambda_{E_2S_2} = 11$, while if the constants assuming equivalent dimer sites are used the corresponding values are $\lambda_{ES} = 1.02$, $\lambda_{E_2S} = 24.6$, and $\lambda_{E_2S_2} = 12$. The solid lines in Figure 4 have been calculated with eq 9 and the parameters assuming nonequivalent binding sites and the dashed lines have been calculated with eq 9 and the parameters assuming equivalent sites.

Temperature-jump experiments with fluorescence detection were carried out at enzyme concentrations of 2–3 μM and total NADH concentrations of 2.5–20 μM with final temperatures of 15.7 and 22.7°. In all cases only a single relaxation process could be detected with a relaxation time very close to that associated with the time constant for heating (~20 μsec). The longest relaxation time, 59 μsec, was observed at an enzyme concentration of 2 μM and an NADH concentration of 2.5 μM, which were the lowest concentrations giving a measurable relaxation amplitude. The relaxation time decreased as the enzyme and NADH concentrations increased, but a quantitative measure of the concentration dependence of the relaxation time was not possible because of the similarity of the chemical and apparatus relaxation times. The enzyme is predominantly a dimer under these conditions and if the observed relaxation time is attributed to the equilibria of eq 3 and 4, assuming equivalent sites, the second-order rate constant for formation of the enzyme–substrate complex can be estimated as $10^9\ M^{-1}\ sec^{-1}$.

Discussion

The binding and fluorescence titration data are consistent with a model for the binding in which NADH binds to the monomer site and both dimer sites of beef heart *s*-malate dehydrogenase with approximately the same affinity. A slight preference for the monomer site and some small degree of cooperativity in binding to the dimer may exist, although the assumption of equivalent sites fits the data quite well. On the other hand, the fluorescent enhancement of NADH is quite different for all of the sites. The exact values for the enhancements vary somewhat depending on the particular model and particular set of data, but in all cases binding to the monomer enhances the fluorescence a very small amount, binding to the first dimer site causes a very large enhancement and the enhancement per bound NADH has an intermediate value between these two extremes when both sites are occupied. The data in Figure 4 show some deviations from the theoretical curves. However, the equilibrium constants used to fit these data were obtained from independent experiments, and small variations

in the constants (within the experimental uncertainties) would improve the fit. Also at high NADH and enzyme concentrations, some nonspecific binding may occur.

The data in Figures 1 and 4 are in good agreement with those of Cassman and King (1972). However, the interpretation proposed here is more consistent with the direct binding data. The explanation for the different fluorescence enhancements is, of course, unknown but could be attributed to protein conformational changes or to structurally nonequivalent sites.

The binding of NADH to porcine *s*-malate dehydrogenase appears to involve two equivalent sites on the dimer (Holbrook and Wolfe, 1972) as in the model proposed here for the beef heart enzyme, with the binding constants being somewhat larger for the porcine enzyme. The temperature-jump data provide indirect evidence for a simple binding mechanism, such as equivalent sites, since only a single relaxation process is observed under conditions where the enzyme is predominantly dimer. A more complex relaxation spectrum would be expected for cooperative binding. The second-order rate constant estimated from the data is close to the maximum possible rate constant for an enzyme–substrate reaction (Alberty and Hammes, 1958).

In summary, the binding of NADH to beef heart *s*-malate dehydrogenase appears to involve nearly equivalent or equivalent enzyme binding sites in both monomer and dimer, with the fluorescence enhancement of NADH depending on the type of site occupied.

Acknowledgment

The authors are indebted to Professor S. Edelstein for several illuminating discussions and to Miss Jean Hsu for her technical assistance.

References

Alberty, R. A., and Hammes, G. G. (1958), *J. Phys. Chem. 62,* 154.
Bray, G. A. (1960), *Anal. Biochem. 1,* 279.
Cantley, L. C., Jr., and Hammes, G. G. (1973), *Biochemistry 12,* 4900.
Cassman, M., and King, R. C. (1972), *Biochemistry 11,* 4937.
del Rosario, E. D. J. (1970), Ph.D. Thesis, Cornell University.
Englard, S., and Breiger, H. A. (1962), *Biochim. Biophys. Acta 56,* 571.
Gerding, R. K., and Wolfe, R. G. (1968), *J. Biol. Chem. 244,* 1164.
Guha, A., Englard, S., and Listowsky, I. (1968), *J. Biol. Chem. 243,* 609.
Holbrook, J. J., and Wolfe, R. G. (1972), *Biochemistry 11,* 2499.
Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175,* 385.
Klingenberg, M. (1963), in Methods of Enzymatic Analysis, Bergmeyer, H. V., Ed., New York, N.Y., Academic Press, pp 528–534.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Silverstein, E. (1970), *Biochim. Biophys. Acta 215,* 205.
Silverstein, E. (1965), *Anal. Biochem. 12,* 199.

Mitochondrial Poly(A) Polymerase from a Poorly Differentiated Hepatoma: Purification and Characteristics†

Kathleen M. Rose,* Harold P. Morris,‡ and Samson T. Jacob*

ABSTRACT: Poly(A) polymerase (EC 2.7.7.19) solubilized from mitochondria of a poorly differentiated rat tumor, Morris hepatoma 3924A, was purified more than 1000-fold by successive column chromatography on phosphocellulose, DEAE-Sephadex, and hydroxylapatite. Purified enzyme catalyzed the incorporation of ATP into poly(A) only upon addition of an exogenous primer. Of several primers tested, synthetic poly(A) was the most effective. The enzyme utilized mitochondrial RNA as a primer at least five times as efficiently as nuclear RNA. The enzyme required Mn^{2+} and had a pH optimum of 7.8–8.2. The enzyme utilized ATP exclusively as a substrate; the calculated K_m for ATP was 28 μM. The polymerization reaction was not inhibited by RNase, ethidium bromide, distamycin, or α-amanitin. The reaction was sensitive to *O-n*-octyloxime of 3-formylrifamycin SV (AF/013). As estimated from glycerol gradient centrifugation and acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the molecular weight of the enzyme was 60,000. The product was covalently linked to the polynucleotide primer and the average length of the poly(A) formed was 600 nucleotides.

Poly(A) polymerase, the enzyme that catalyzes the incorporation of adenylic acid residues into poly(A), is widely distributed in nature. Thus, the enzyme is present in a variety of bacteria (August et al., 1962; Gottesman et al., 1962; Hardy and Kurland, 1966; Payne and Boezi, 1970; Colvill and Terzi, 1968; Ohasa and Tsugita, 1972; Modak and Sri-

† From the Department of Pharmacology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033. *Received June 4, 1974.* Supported by a U.S. Public Health Service Grant, CA 15733, from The National Cancer Institute.
‡ Department of Biochemistry, Howard University College of Medicine, Washington, D.C.