Allosteric and Nonallosteric Interactions with Reduced Nicotinamide Adenine Dinucleotide in Two Forms of Cytoplasmic Malic Dehydrogenase†

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ABSTRACT: Two forms of cytoplasmic malic dehydrogenase have been isolated from beef heart. One form (dehydrogenase b) shows a protein concentration dependence in the binding of NADH, and cooperative kinetics with respect to NADH, and has fructose 1,6-bisphosphate as an allosteric inhibitor. The second form (dehydrogenase a) has a specific activity twice that of dehydrogenase b, exhibits no cooperativity of NADH binding and kinetics, and is unresponsive to the addition of fructose 1,6-bisphosphate. Dehydrogenases a and b have identical amino acid composition and subunit molecular weight. They both have the dimer as the maximal degree of polymerization. Preliminary indications suggest that dehydrogenase a exists in a monomer–dimer equilibrium with an association constant smaller than that found for dehydrogenase b. The major structural distinction between the two forms is that dehydrogenase a preparations contain 1.3–1.8 mol of trichloroacetic acid precipitable phosphate/mol of enzyme, while dehydrogenase b preparations contain 0.3–0.6 mol of phosphate/mol of enzyme. It is suggested that the presence of phosphorylated and nonphosphorylated forms of dehydrogenase may reflect a metabolic control mechanism similar to that known for several regulatory enzymes.

Recent studies on cytoplasmic malic dehydrogenase, isolated from beef heart, have demonstrated cooperative binding by NADH[1] and provided evidence for fructose 1,6-bisphosphate as an allosteric modifier (Cassman and King, 1972a; Cassman, 1973). On the basis of these results, it was suggested that cytoplasmic malic dehydrogenase may have a regulatory function, possibly in controlling the mode of oxidation of glycolytic NADH (Cassman, 1973).

In this publication, we report experiments which show that malic dehydrogenase can be purified in two forms which differ in their interaction with NADH. The active form showing no allosteric interactions with NADH is termed cytoplasmic malic dehydrogenase a (dehydrogenase a) and the less active cooperative species is termed dehydrogenase b, consistent with the nomenclature introduced by Cori and Green (1943) for phosphorylase. The two forms of the enzyme have identical amino acid compositions and subunit molecular weight, but differ in the amount of covalently bound phosphate.

The existence of two stable enzyme species with different regulatory properties has been observed with a number of enzymes involved in metabolic control (Holzer and Duntze, 1971). The observation of a similar phenomenon with cytoplasmic malic dehydrogenase lends further credence to the hypothesis that this enzyme plays a significant role in metabolic regulation.

Experimental Section

Materials. NADH and oxaloacetate, both A grade, were obtained from Calbiochem. Fructose 1,6-bisphosphate was the tetrasodium salt prepared by Sigma. Snake venom phosphodiesterase was the product of Worthington. Sodium dodecyl sulfate was obtained from Sigma and twice recrystallized before use. D_2O (99.8%) was obtained from Thompson-Packard. All other chemicals were standard reagent grade.

Enzyme Assay. Standard assays were performed in 0.1 M triethanolamine (pH 7.4) using 1×10^{-4} M oxaloacetate and 1×10^{-4} M NADH. Measurements were made at 360 nm, using a Zeiss PMQ-II spectrophotometer. One unit of enzyme activity is defined as a decrease of 0.010 optical density (OD) unit/min. Protein concentrations were determined by the method of Lowry *et al.* (1951) or from the absorbance at 280 nm. Values of $A_{280}^{1\%}$ for both forms of the enzyme are given in Table III.

Binding and Kinetic Measurements. All fluorescence measurements were made on a Hitachi Perkin-Elmer MPF-2A fluorescence spectrometer, equipped with a thermostated cell holder. The temperature was kept constant at 21–22°, using a circulating water bath.

Determinations of NADH–enzyme interactions were made by following either the enhancement of NADH fluorescence at 420 nm or the quenching of protein fluorescence at 340 nm. Calculations of fractional saturation and free NADH concentration for generating the Scatchard plots were made as described previously (Cassman and King, 1972a).

The rate of NADH oxidation in the presence of oxaloacetate was followed fluorimetrically. Measurements of NADH fluorescence were made at 420 nm following excitation at 340 nm. All reactions were performed in 3-ml volumes, and changes in fluorescence intensity were followed on a strip chart recorder. Measurement of initial rates was begun within 20 sec after addition of the enzyme to the reaction mixture. The rate of decrease in fluorescence intensity was linear for at least the first minute of measurement. The NADH fluorescense intensity was calibrated with a series of NADH solutions of known concentrations, which had been diluted from a stock solution. The concentration of the stock solution was determined by measuring the optical density at 340 nm and

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[1] Abbreviations used are: NADH, reduced nicotinamide adenine dinucleotide; $\bar{\nu}$, average moles of NADH bound per mole of enzyme (mol wt 77,000).

applying the extinction coefficient $\epsilon_{340\ nm}^{1\ cm} = 6.22 \times 10^3$ M^{-1} cm^{-1} (Horecker and Kornberg, 1949). The dependence of fluorescence intensity on NADH concentration was linear over the concentration range used.

Polyacrylamide Gel Electrophoresis. Electrophoresis on polyacrylamide gels containing 0.1% sodium dodecyl sulfate was performed according to Weber and Osborn (1969). Prior to electrophoresis the enzymes were incubated at 37° in 0.1 M potassium phosphate (pH 7.0), containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol, for 2 hr to allow dissociation. The gels were stained with Coomassie Brilliant Blue and destained electrophoretically.

Amino Acid Analysis. Protein samples containing 2–3 mg of protein were dried *in vacuo* and then hydrolyzed (6 N HCl, *in vacuo*, 20 hr, 110°). The hydrolysate was filtered and evaporated to dryness. The residue was taken up in water and dried, three successive times. Finally, it was taken up in a measured volume of 0.2 M sodium citrate buffer (pH 2.2). The hydrolysate was chromatographed using the Beckman Model 120C amino acid analyzer, according to the method of Spackman *et al.* (1958).

Ultracentrifuge Methods. A Spinco Model E analytical ultracentrifuge equipped with an electronic speed control was employed for all sedimentation studies. Equilibrium ultracentrifugation was performed using 12-mm light path cells fitted with sapphire windows. Column heights of 3 mm were used in these studies. Prior to centrifugation, the samples were dialyzed for 24 hr in the cold against two changes of 100-fold their volume of D_2O or H_2O buffers. A sample of the dialysate was then used as the reference solvent. Solvent densities were determined in triplicate in 5-ml pycnometers (Schachman, 1957). Measurements were made using a Rayleigh optical system and Eastman Kodak II-G spectroscopic plates.

Calculations of molecular weight in D_2O were made using the equation (Edelstein and Schachman, 1967)

$$kM(1 - (\bar{v}/k)\rho) = (2RT/\omega^2)(\mathrm{d} \ln c/\mathrm{d}r^2)$$

where k is the ratio of the molecular weight in the deuterated to that in the nondeuterated solvent. The value of k was taken as 1.0155 (Martin *et al.*, 1959; Hvidt and Nielson, 1966). All other measurements and subsequent data analysis were as previously described (Cassman and King, 1972a).

Sedimentation velocity studies were performed at 56,000 rpm in double sector 12-mm light path cells, fitted with quartz windows. Temperatures were maintained constant at 17–20°.

Partial specific volumes were calculated from the amino acid composition by the method of Cohn and Edsall (1943).

Phosphate Analysis. Phosphate was determined by the method of Sumner (1944). The procedure was scaled down so that the digestion and color development could be performed in 1.2-ml calibrated microdigestion flasks. Under these conditions, as little as 0.01 μmol of phosphate could be determined.

Snake Venom Phosphodiesterase Digestion. Incubation of 2–4 mg of enzyme was performed at 37° for 3 hr in 10 ml of 0.1 M Tris buffer (pH 9.0) plus 5×10^{-3} M $MgCl_2$, according to the method of Shapiro *et al.* (1967). The enzyme was separated from low molecular weight products on a Bio-Rad P-2 column.

Results

Enzyme Purification. Beef heat cytoplasmic malic dehydrogenase was prepared according to the method of Guha *et al.* (1968). Purified preparations fell into two distinct categories which could be distinguished by several criteria of binding and kinetic behavior. The two forms could be most easily recognized by the differences in the specific activities under standard assay conditions, and by the A_{280}/A_{260} ratios. The specific activities for dehydrogenase a were in the range 75,000–110,000, while dehydrogenase b gave values of 40,000–55,000. The ratio of A_{280}/A_{260} for dehydrogenase a was 1.7–1.8 and 1.4–1.55 for dehydrogenase b.

Examination of these apparently homogeneous preparations indicated the presence of two kinds of low molecular weight contaminants. The relatively low A_{280}/A_{260} value for dehydrogenase b was due to the presence of a tightly bound low molecular weight material having an absorption maximum at 257–262 nm. A peak in this region could be observed by obtaining a difference spectrum of dehydrogenase b *vs.* dehydrogenase a. Although this material could not be removed by repeated dialysis, or by ammonium sulfate precipitation, it did not appear to be covalently bound, since it could be released upon precipitation of the enzyme with cold 10% trichloroacetic acid. It could also be released by incubation with venom phosphodiesterase. When the incubation mixture was passed through a P-2 column, the 280/260 ratio of the eluted dehydrogenase b increased to 1.6–1.7. Comparison with dehydrogenase a now showed no difference spectrum. The phosphodiesterase-treated enzyme was identical with untreated sample in all other physical and functional properties tested. The 260-nm absorbing substance did not appear to be NAD^+ or $NADP^+$, since dehydrogenase b preparations did not show a visible fluorescence in strong alkali (Kaplan *et al.*, 1951). No attempt was made to further characterize the material.

Both dehydrogenase a and b preparations showed small variable amounts of absorption between 320 and 420 nm. This could be removed by dialysis against high ionic strength phosphate buffers. The recovered enzyme was in all other respects identical with the original preparation. Cold trichloroacetic acid precipitation of samples having such absorption yielded a supernatant which developed an absorption band at 332 nm when made strongly alkaline. The spectral properties of this material are characteristic of pyridoxol derivatives (Johnson and Metzler, 1970). This is probably a contaminant from a transaminase activity which is copurified up to the last step of the isolation procedure (unpublished data). The purified enzyme preparation itself contained no transaminase activity.

Kinetic and Binding Properties of Dehydrogenases a and b. The two enzyme forms showed differences in their mode of interaction with NADH when studied both by equilibrium binding and by kinetic methods. Initial rate studies of NADH oxidation were performed at a fixed oxaloacetate concentration of 10^{-5} M and varying NADH concentrations. The results are shown in Figure 1, presented as double-reciprocal plots of 1/velocity *vs.* 1/[NADH]. The curves represent the best least-squares fit to the data, using either a linear or quadratic function. The program used was a standard polynomial regression analysis from the University of California, Los Angeles, Biomedical Computer Program series. The choice of a linear or quadratic fit was made by selecting the function which gave the smallest variance, arising from random error, for the fit of the least-squares line to the points. In the case of dehydrogenase a, where both functions gave equally good fits, the linear regression was chosen. The plot for dehydrogenase b, however, was fit substantially better by a nonlinear regression. In this case, the best linear fit required that the high and low

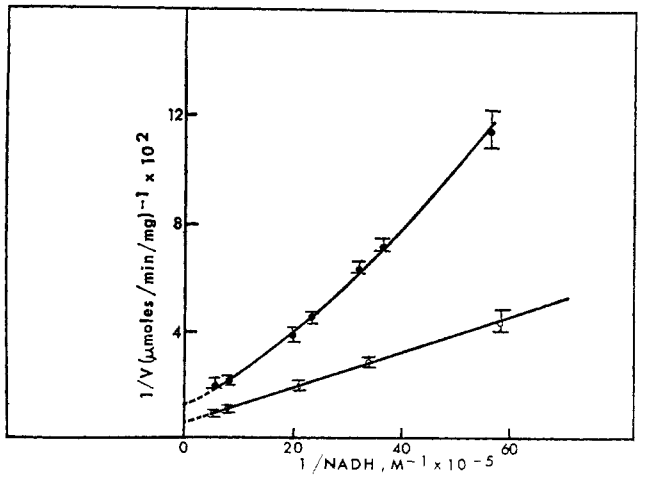

FIGURE 1: Double-reciprocal plot of the reaction rate of dehydrogenases a and b as a function of NADH concentration. Measured at 1×10^{-5} M oxaloacetate, in sodium potassium phosphate (pH 6), ionic strength 0.05. The ordinate is the reciprocal of the initial velocity of the reaction, in units of micromoles of NADH oxidized per minute per milligram of protein. The abscissa is the reciprocal of the molar concentration of NADH. The error bars represent ±standard deviation: (○) dehydrogenase a; (●) dehydrogenase b.

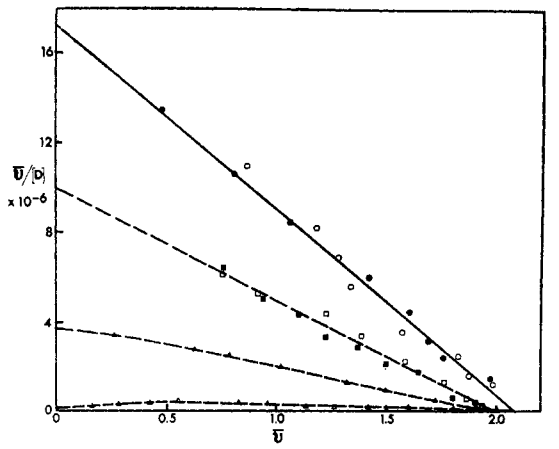

FIGURE 2: Scatchard plots of NADH binding by dehydrogenases a and b. Titrations with NADH were performed by following either the quenching of protein fluorescence or the enhancement of NADH fluorescence. The buffer used was sodium potassium phosphate (pH 6.0), ionic strength 0.05. The symbols represent experimental points obtained at different enzyme concentrations. Dehydrogenase a (solid line): (●) 0.08 mg/ml of dehydrogenase; (○) 0.008 mg/ml of dehydrogenase b (broken lines): (□) 0.0008 mg/ml of dehydrogenase; (■) 0.0024 mg/ml of dehydrogenase; (▲) 0.008 mg/ml of dehydrogenase; (△) 0.24 mg/ml of dehydrogenase.

concentration points fall above the line, and intermediate concentrations below the line, rather than showing random deviations. The upward curvature in the plot for dehydrogenase b is characteristic of systems showing positive cooperativity. Although this is consistent with other allosteric properties associated with the enzyme, one cannot exclude the possibility that the curvature of the double-reciprocal plot arises from a steady-state kinetic mechanism which includes more than one significant path for NADH binding (Sweeny and Fisher, 1968).

The marked differences in the qualitative kinetic behavior of dehydrogenases a and b is accompanied by a more than twofold higher apparent V_{max} for dehydrogenase a, compared to dehydrogenase b (Figure 1).

The difference in the enzyme interactions with NADH could also be demonstrated by equilibrium binding techniques. The binding of NADH as a function of enzyme concentration is given in Figure 2. Dehydrogenase a produced overlapping linear Scatchard plots at all enzyme concentrations studied. This indicated the presence of noninteracting, identical binding sites, with an equilibrium constant which is independent of enzyme concentrations. In contrast, dehydrogenase b generated Scatchard plots which exhibit a progressive decrease in the apparent affinity for NADH with increasing enzyme concentration.

It should be noted that both enzymes have nearly identical abscissa intercepts in the Scatchard plots, indicating a stoichiometry of 2 mol of NADH/mol of enzyme for both dehydrogenases a and b. However, the values for the ordinate intercepts indicate an NADH dissociation constant of 1.16×10^{-7} M for dehydrogenase a compared to a value of about 2×10^{-7} M for dehydrogenase b at low enzyme concentrations.

The kinetic and binding data for dehydrogenase b showed an apparent discrepancy, since the cooperativity of binding in this system was dependent on protein concentration, and became negligible at enzyme concentrations where the kinetic data showed a marked cooperativity. These differences may result from the presence of the cosubstrate for the enzymatic reaction, oxaloacetate. Further kinetic analyses being performed in our laboratory appear to confirm this hypothesis (D. Vetterlein and M. Cassman, manuscript in preparation).

The protein concentration dependence of the binding curves for dehydrogenase b has been taken to reflect an equilibrium between a monomer and a cooperative dimer. A detailed study of this behavior at pH 7 has been reported (Cassman and King, 1972a). An alternative model would involve an equilibrium between a monomer and a noncooperative dimer with a lower affinity for NADH than the monomer. The two models can be discriminated largely through differences in the Scatchard plot above 90% and below 10% fractional saturation. Although the measurements of fractional saturation are reproducible to 5–7%, the Scatchard parameter $\bar{v}/[D]$ is very sensitive to small errors in $\bar{v}$ at high saturations, and to small errors in free ligand concentration at low saturations. Both models would, however, be expected to show an apparent cooperativity of NADH binding in the region of enzyme concentration where both monomer and dimer coexist (Frieden, 1967; Cassman and King, 1972a). The degree of cooperativity, reflected by a curvature convex to the abscissa in a Scatchard plot, would be relatively small for both models when the dimer concentration is low. The slight curvature expressed at 0.008 and 0.24 mg/ml of dehydrogenase b might therefore be expected from either of the hypothesized models. In addition it is felt that the precision of the measurements permits the points to be fit to a curve rather than a straight line, particularly at 0.24 mg/ml.

Finally, dehydrogenases a and b differ in their response to fructose 1,6-bisphosphate. Fructose 1,6-bisphosphate has been shown to be an allosteric inhibitor of NADH binding to dehydrogenase b (Cassman, 1973). Binding of NADH to dehydrogenase a, however, is unaltered by concentrations of up to 0.1 M fructose 1,6-bisphosphate (Figure 3).

Electrophoretic Comparison of Dehydrogenases a and b. Electrophoresis of dehydrogenase a or b on polyacrylamide gels containing 0.1% sodium dodecyl sulfate resulted in the formation of a single sharp band. When electrophoresed together, a and b also tracked as a single band, with a mobility identical with that observed when the enzymes were examined

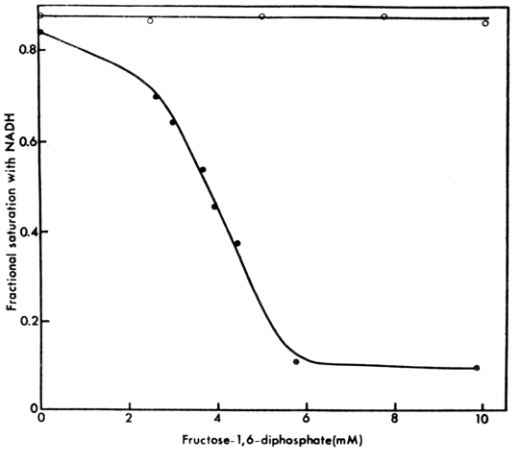

FIGURE 3: Effect on NADH binding of added fructose 1,6-bisphosphate. The NADH–enzyme interaction was followed by measuring the quenching of protein fluorescence at 340 nm upon excitation at 280 nm. Fructose 1,6-bisphosphate alone had no effect on the enzyme fluorescence. Titrations were performed in sodium potassium phosphate (pH 6.0), ionic strength 0.05. The enzyme concentration was 5 μg/ml. The NADH concentration was 8×10^{-7} M for dehydrogenase a and 1.2×10^{-6} M for dehydrogenase b: (○) dehydrogenase a; (●) dehydrogenase b.

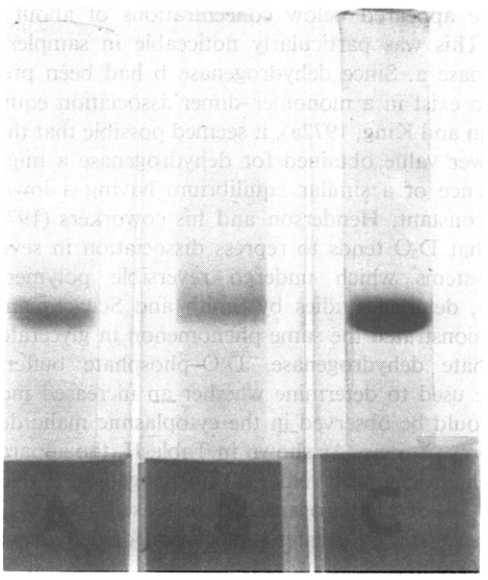

FIGURE 4: Comparison of dehydrogenase a and b on acrylamide gels in the presence of 0.1% sodium dodecyl sulfate; 20 μg of each enzyme was used: (A) dehydrogenase a; (B) dehydrogenase b; (C) dehydrogenase a + dehydrogenase b. The direction of migration is from the top of the photograph.

independently (Figure 4). This suggests that each of these enzyme forms has only one kind of subunit, and that this subunit has the same molecular weight in both forms. This mol wt is 38,000 ± 1000, obtained from previous equilibrium ultracentrifuge measurements in guanidine hydrochloride (Cassman and King, 1972b).

Amino Acid Composition. Amino acid analyses were carried out on both forms of the enzyme. The results indicate an essentially identical amino acid composition for dehydrogenases a and b (Table I).

These data are generally in excellent agreement with the previously published results of Siegel and Englard (1962), on a preparation also obtained from beef heart. However, they differ significantly in the values for serine and threonine, perhaps due to experimental error. Insufficient evidence is available to determine which form of the enzyme was isolated in this study.

Ultracentrifuge Analysis. Sedimentation equilibrium measurements in sodium potassium phosphate (pH 6.7), ionic strength 0.05, gave a mol wt for dehydrogenase b of 77,000 ± 1000. However, similar studies showed a consistently lower value of about 10% for the mol wt of dehydrogenase a in H_2O–phosphate buffer, compared to dehydrogenase b (Table II). The plots of ln c *vs.* r^2 were measured over a concentration range of 0.1–1.0 mg/ml. Although the measured points appeared linear over most of this concentration span, a slight

TABLE I: Amino Acid Composition of Dehydrogenases a and b.[a]

	b (Residues/1000)	a (Residues/1000)
Lys	106	97
His	18	15
Arg	30	31
Asp	109	117
Thr	47	54
Ser	72	71
Glu	93	91
Pro	39	41
Gly	76	73
Ala	100	96
Cys	12	14
Val	76	76
Met	24	24
Ile	53	55
Leu	87	91
Tyr	23	23
Phe	35	31

[a] Results averaged from two separate hydrolyses and analyses of each of the two enzyme species. The averaged values were rounded off to the nearest integer. The duplicate analyses differed by 10% or less.

TABLE II: Molecular Weights of Dehydrogenases a and b in H_2O and D_2O.[a]

	a	b
H_2O	71,000 ± 2000	77,200 ± 1000
D_2O	78,500 ± 1000	77,400 ± 2000

[a] Experiments were performed in 0.01 M phosphate buffer (pH 6.7) and either in H_2O or 99.8% D_2O. The values obtained are averaged from two–three measurements at sedimentation equilibrium, performed at 24,000–32,000 rpm. In some cases, curvature of the ln c *vs.* r^2 plots was observed below 0.2–0.3 mg/ml. In these circumstances, the molecular weights were calculated from the linear concentration range between 0.4 and 1.0 mg/ml. The partial specific volume was calculated as 0.745 from the amino acid composition.

curvature appeared below concentrations of about 0.2–0.3 mg/ml. This was particularly noticeable in samples of dehydrogenase a. Since dehydrogenase b had been previously shown to exist in a monomer–dimer association equilibrium (Cassman and King, 1972a), it seemed possible that the somewhat lower value obtained for dehydrogenase a might be a consequence of a similar equilibrium having a lower association constant. Henderson and his coworkers (1970) have shown that D_2O tends to repress dissociation in several enzyme systems which undergo reversible polymerization. Recently, detailed studies by Smith and Schachman (1973) have demonstrated the same phenomenon in glyceraldehyde-3-phosphate dehydrogenase. D_2O–phosphate buffers were therefore used to determine whether an increased molecular weight could be observed in the cytoplasmic malic dehydrogenase preparations. As shown in Table II, the apparent mol wt of dehydrogenase b remained unchanged at 77,000, while the mol wt of dehydrogenase a increased from 71,000 to 78,500, suggesting a greater dissociation of dehydrogenase a in the H_2O buffer.

Sedimentation velocity studies were performed with both dehydrogenases a and b in the H_2O–phosphate buffer used for the equilibrium measurements. A single symmetrical peak was obtained for both preparations, with an $s_{20,w}$ of 4.6–4.7 S at concentrations of 2–3 mg/ml. This value is consistent with a globular protein of mol wt ~80,000.

The ultracentrifuge studies, in conjunction with the analysis of subunit composition given above, indicate that a dimer of mol wt 77,000–78,000 represents the largest molecular weight species generated by either form of the native, unliganded enzyme. The apparent difference in the monomer–dimer association constants, inferred from the H_2O–D_2O data, therefore seems to represent the only significant difference between the two enzymes, with respect to the molecular weight species present in solution. Further measurements over a wider concentration range will be required to confirm this apparent variation in association constants.

Phosphate Content. Since a number of regulatory enzymes are known to be modified by phosphorylation, the two enzyme forms of cytoplasmic malic dehydrogenase were analyzed for trichloroacetic acid precipitable phosphate.

Dehydrogenases a and b were extensively dialyzed against cold distilled water, prior to precipitation by cold 10% trichloroacetic acid. They were then washed twice with 10% trichloroacetic acid, and the pellets were digested in H_2SO_4 and analyzed for phosphate. Results from four different isolations of both enzyme forms gave values of 1.3–1.8 mol of phosphate/mol of dehydrogenase a, and 0.3–0.6 mol of phosphate/mol of dehydrogenase b. To ensure that none of the measured phosphate was due to trapped noncovalently bound material, an additional analysis was performed on samples to which 0.1 M phosphate was added. These samples were precipitated with 10% trichloroacetic acid, washed, and redissolved in 0.1 N NaOH twice, prior to the phosphate analysis. The samples treated in this manner gave results identical with those described above.

These results indicate the presence of covalently bound phosphate. Since both the pyridoxol contaminant and the 260-nm absorbing moiety of dehydrogenase b are released by trichloroacetic acid precipitation (see section on Enzyme Purification under Results), these components cannot contribute to the results obtained. Prior treatment of both dehydrogenases a and b with venom phosphodiesterase did not affect the values obtained for trichloroacetic acid precipitable phosphate.

Discussion

Cytoplasmic malic dehydrogenase has been purified from beef heart in two forms which can be distinguished by the manner in which they interact with NADH. A comparison of some properties of the two species is given in Table III. Dehydrogenase a shows no allosteric properties in its interactions with NADH. Dehydrogenase b exhibits a concentration dependence of the binding, and cooperative kinetics, with respect to NADH. Dehydrogenase b also displays an inhibition of NADH binding by an allosteric inhibitor, fructose 1,6-bisphosphate.

The functional differences between dehydrogenases a and b appear to be attributable to subtle differences in structure. Identical results for both dehydrogenases a and b from electrophoresis in conjunction with previous ultracentrifuge studies in guanidine hydrochloride (Cassman and King, 1972b) indicate that both enzymes have a single common subunit of mol wt 38,000 ± 1000. Similarly, sedimentation velocity and sedimentation equilibrium experiments suggest that the maximum degree of polymerization of both enzymes is a dimer of mol wt 77,000–78,000.

Studies of binding of NADH to dehydrogenase b, as well as sedimentation equilibrium measurements, had previously indicated that this enzyme form existed in a monomer–dimer equilibrium (Cassman and King, 1972a). This equilibrium was reflected in the binding data by a protein concentration dependence of the cooperativity of NADH binding, where dehydrogenase b showed marked cooperativity at high enzyme concentrations, but approached a noninteracting system as the enzyme concentration decreased. Although dehydrogenase a shows no cooperativity of NADH binding at any protein concentrations tested, the results of the ultracentrifuge studies in H_2O and D_2O indicate that this enzyme form may also undergo an association–dissociation equilibrium. The apparent increased association of dehydrogenase b, with identical solvent conditions and protein concentrations as dehydrogenase a, may be a reflection of the same quaternary structure constraints which generate the changes in the hydrogenase b dimer.

A major difference between the two enzyme forms is the amount of covalently bound phosphate. Dehydrogenase a contains 0.65–0.9 mol of phosphate/subunit, while dehydrogenase b contains 0.15–0.3 mol of phosphate/subunit. In the

TABLE III: Comparison of Dehydrogenases a and b.

	a	b
Sp act.[a]	75,000–110,000	40,000–55,000
A_{280}/A_{260}	1.7–1.8	1.4–1.55
$A_{280}^{1\%}$	9.1–9.3	10.3–10.7
$s_{20,w}$ (S)[b]	4.6–4.7	4.6–4.7
Mol wt[c]	77,000–78,000	77,000–78,000
Cl_3CCOOH-precipitable phosphate[d] (mol of phosphate/mol of protein)	1.3–1.8	0.3–0.6

[a] Measured in 0.01 M triethanolamine buffer (pH 7.4) at 10^{-4} M NADH and 10^{-4} M oxaloacetate. [b] Measured at 2.0–3.0 mg/ml of protein. [c] Obtained from sedimentation equilibrium experiments in 0.01 M phosphate (pH 6.7) and D_2O as a solvent, at concentrations above 0.4 mg/ml. [d] Calculated assuming a mol wt of 77,000 for both proteins.

absence of evidence for a parallel phosphorylation–dephosphorylation with enzyme activation–inactivation, it is not possible to unequivocally show that the difference in phosphate content is causally related to the functional differences in the two enzyme forms. However, there is good reason to suspect this as being the case, since there is ample precedent for phosphorylation–dephosphorylation as a mechanism for regulating enzyme activity (Holzer and Duntze, 1971).

The small amount of trichloroacetic acid precipitable phosphate found in preparations of dehydrogenase b, and the lower than stoichiometric ratios found in preparations of dehydrogenase a, may be due to heterogeneity with respect to the two forms of cytoplasmic malic dehydrogenase. It is not yet clear what governs the interconversion of these two enzyme species in beef heart, but it seems *a priori* unlikely that in any given sample all of the cytoplasmic malic dehydrogenase will be in one or the other form. Assuming 0 mol of phosphate/mol of dehydrogenase b and 2.0 mol of phosphate/mol of dehydrogenase a, there is a maximum 30% heterogeneity in some of the preparations examined.

A possible role of cytoplasmic malic dehydrogenase in the reoxidation of cytoplasmic NADH has been proposed by Krebs and his coworkers (1967). The subsequent entry of malate into the mitochondrion would then allow these reducing equivalents to be coupled to oxidative metabolism. It is, however, necessary to account for the observed variation, under different physiological conditions, of reducing equivalents from glycolytic NADH appearing as lactate or oxidized in the mitochondrion. The cooperative binding of NADH and allosteric inhibition by fructose bisphosphate observed with dehydrogenase b prompted the suggestion that cytoplasmic malic dehydrogenase may also function as a regulatory point for the flow of cytoplasmic NADH (Cassman, 1973). The fructose bisphosphate effect provided an apparently reasonable mechanism for such control, since changes in fructose bisphosphate levels vary with the rate of glucose utilization and lactate production in a manner consistent with this model (Williamson, 1966). The existence of two stable forms of the enzyme showing differences in the cooperative interactions with NADH is further evidence that the enzyme fulfills some regulatory role in the cell. However, the role of fructose bisphosphate has become more ambiguous. The significance of the fructose bisphosphate response was predicted on the absence of cooperativity of NADH binding at low enzyme concentrations, and the induction of an altered binding curve by fructose bisphosphate at these enzyme levels. Kinetic experiments have shown that dehydrogenase b in the presence of oxaloacetate, and in the absence of fructose bisphosphate, exhibits some cooperativity even at low enzyme levels. It is clear that the *in vivo* significance of the fructose bisphosphate response will require a more complete understanding of the enzyme mechanism *in vitro*.

Although the nature of the allosteric effects is consistent with an involvement of the enzyme in controlling the flow of glycolytic NADH, other physiological roles cannot be excluded. The suggested participation of cytoplasmic malic dehydrogenase in several metabolic processes (Lardy *et al.*, 1964; Krebs *et al.*, 1967; Marco and Sols, 1969) leaves open the possibility that the enzyme might function as a control point for more than one metabolic pathway. The assignment of an exclusive *in vivo* regulatory role for the enzyme is clearly premature at this point.

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