Vol. 82, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 14, 1978 Pages 943-950

THE RELATION OF THE pH AND CONCENTRATION-DEPENDENT DISSOCIATION OF PORCINE HEART MITOCHONDRIAL MALATE DEHYDROGENASE

David C. Wood, C. Thomas Hodges and John H. Harrison

Kenan Laboratories of Chemistry
University of North Carolina
Chapel Hill, N. C. 27514

Received April 13,1978

SUMMARY

The relationship of the pH-dependent and concentration-dependent dissociation of porcine heart mitochondrial malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) was investigated by means of gel filtration chromatography utilizing a standardized Sephacryl S-200 column. The results obtained indicate that the dimeric form of this enzyme dissociates to yield monomers at conditions of low protein concentration or at pH values below neutrality. In addition it is apparent that as the pH is lowered, the minimum concentration of protein required to maintain the enzyme in the dimeric form is increased.

INTRODUCTION

Numerous enzymes have been shown to exist as multisubunit complexes in which the subunits are chemically identical. Several of these have been shown to undergo pH-dependent and/or concentration-dependent dissociations (1-5). If such dissociations are unknown or not considered, results obtained from experiments requiring homogeneity of quaternary structure can be drastically misinterpreted. Studies of this nature include investigations of the cooperativity of coenzyme and substrate binding. Often these studies are conducted using extremely low protein concentrations in order to accurately determine the coenzyme and substrate dissociation constants and stoichiometry. These low concentrations could lead to unnoticed protein dissociation and therefore ambiguous interpretations of results.

Porcine heart mitochondrial malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) has recently been shown to exhibit both a concentration

0006-291X/78/0823-0943$01.00/0

and a pH-dependent dissociation (4,5). At concentrations of 114 μM the enzyme dissociates as the pH is lowered below 6.0 with an apparent pK_a of 5.3. At pH 7.0 the enzyme is in its dimeric form at a concentration of 30 μM and is fully dissociated at 0.2 μM (4). This paper reports the results of experiments relating the pH-dependent dissociation of malate dehydrogenase to the concentration dependent dissociation of the enzyme. The implications of this relationship to previous studies (6,7) dealing with the cooperativity of NADH binding to malate dehydrogenase is discussed.

Experimental

Materials: Mitochondrial malate dehydrogenase was purified as previously described by Gregory et al. (8). Oxalacetate, NADH, AMP, adenosine, adenine, L-malic acid and Sephacryl S-200 were purchased from Sigma Chemical Company.

Sample Preparations: Enzyme samples were dialyzed versus 50 mM sodium phosphate buffer (pH 7.5, 6.8, 5.8 or 5.0) at 4° prior to protein concentration determinations. Protein concentrations were determined on a Pye-Unicam SP-800 Spectrophotometer using the previously reported extinction coefficient $E^{1\%}_{1cm} = 2.53$ (8). Protein samples were diluted to the desired concentration with the appropriate buffer and applied to the column in a volume of 2 ml.

Gel Filtration Chromatography: Gel Filtration chromatography was performed using a Sephacryl S-200 column (2.5 x 100 cm). The column was equilibrated with either 50 mM sodium phosphate or 50 mM Tris-acetate buffer at the appropriate pH in the absence or presence of substrate or coenzyme. Prior to use the Tris-acetate buffer was passed over a Chelex-100 column in order to remove heavy metal ions. The column was operated in reverse flow with the flow rate maintained at approximately 60 ml/hr by a Buchler Polystaltic pump. Appropriate proteins of known molecular weight were used to calibrate the column (4). The apparent molecular weight of malate dehydrogenase under various conditions was determined by its elution volume from the calibrated column. Protein elution was monitored at 230 nm on a Unicam SP 800 spectrophotometer. When protein concentrations were below detection limits by absorption measurements, protein elution volume was determined by enzymatic assay. A range of 45 ml of elution volume from the Sephacryl S-200 gel chromatography column was observed between the dimer and fully dissociated enzyme. Approximately a 20-fold dilution in protein concentration was observed for peak fractions. This value was assumed to be constant for each determination.

Enzymatic Assay: Enzymatic assays of samples eluted from the Sephacryl S-200 column were performed by addition of 2.8 ml of collected fractions to cuvettes containing 0.1 ml of 150 mM oxalacetate and 0.1 ml of 4.5 mM NADH at the pH under investigation. The decrease in absorption at 340 nm was followed upon the addition of the aliquot from the eluted fractions on the spectrophotometer described above in a cell compartment thermostatted at 25°. All fractions were warmed to 25° in a water bath immediately prior to assay.

Results and Discussion

The observed apparent molecular weight of porcine heart mitochondrial

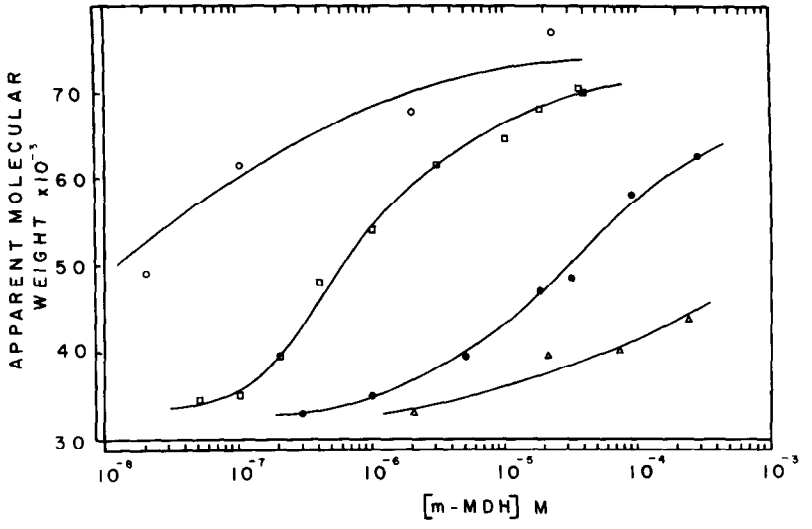

Figure 1. Effect of pH on the concentration-dependent dissociation of malate dehydrogenase. Mitochondrial malate dehydrogenase at the concentration indicated was dialyzed versus 50 mM sodium phosphate buffer adjusted to the appropriate pH. The protein sample was applied to a Sephacryl S-200 column equilibrated in the same buffer at the pH indicated: (△) 5.0; (●) 5.8; (□) 6.8; (○) 7.5. The apparent molecular weight was determined as described under "Experimental".

malate dehydrogenase as a function of protein concentration and pH in 50 mM sodium phosphate buffer is indicated in Figure 1. The enzyme has a tendency towards dissociation at conditions of low pH or at low protein concentrations. At higher concentrations or at pH values above neutrality, the enzyme tends to occur as a dimer. At pH 5.0, malate dehydrogenase exhibits an apparent molecular weight suggestive of a fully dissociated enzyme even at protein concentrations as high as 200 μM (14 mg/ml). By contrast, at a pH value of 7.5 the enzyme appears to be fully in the dimeric form even at a protein concentration as low as 0.1 μM. Recently Frieden et al. (9) have reported the lack of evidence for dissociation of this enzyme as determined by stop-flow kinetic analysis at pH 7.5 in sodium phosphate buffer. In view of the data presented in Figure 1 it is evident that little or no dissociation would be apparent under these conditions. However a change in the value of V_{max} under these conditions from 425 s^{-1} to 675 s^{-1} was observed by these authors over a protein concentration

range of 3×10^{-3} μM to 1.14 μM. This change in kinetic constants may in fact be correlated with the small but significant change in observed apparent molecular weight (70,000 to 50,000) at pH 7.5 as determined in this work. Furthermore as indicated in Figure 1 as the pH is lowered, the minimum concentration of protein required to maintain the enzyme in the dimeric form is increased. Utilizing the data determined at pH 6.8, an apparent dissociation constant of 0.7 μM was determined. This value is in good agreement with that value (0.2 μM) determined by Shore and Chakrabarti (3) by means of fluorescence polarization at pH 8.0 and 25° in 50 mM Tris-acetate buffer. The value determined by this method is for comparison only and represents a constant for dissociation in the chromatography column under conditions of a 20-fold dilution during elution of the enzyme and hence must not be strictly interpreted as a true dissocistion constant. Note that the concentration values given in Figure 1 are the concentration of the enzyme sample before chromatography. It is evident from the data presented in Figure 1 that dissociation of the enzyme occurs under certain conditions of pH and protein concentration, whether or not this dissociation is in anyway related to the kinetic characteristics of the active enzyme has not been addressed in this work.

The data in Table I indicate that partial reassociation of the subunits is facilitated by the presence of either substrate or by adenosine (a component part of NAD^+). As indicated in Table I both oxalacetate (0.2 mM) and L-malate (1.0 mM) were observed to facilitate the formation of dimers in malate dehydrogenase under conditions in which the protein is normally found to be a monomer of approximately 35,000 molecular weight. In both instances the concentration of substrate used is well above their published K_m values, 18 μM and 110 μM for oxalacetate and malate respectively (10). This observation is in agreement with the effect of substrates upon the pH-dependent dissociation of malate dehydrogenase previously reported by this laboratory (5). Previous gel chromatography studies have indicated that both NAD^+ and NADH are capable of shifting both the pH and concentration-dependent dissociation equilibria of this enzyme in favor

Table I. Apparent molecular weight determinations of malate dehydrogenase in the presence of substrates and constituent parts of NAD^+.

Mitochondrial malate dehydrogenase was applied to a Sephacryl S-200 column (2.5 x 100 cm) at a concentration of 0.1 μM in 50 mM sodium phosphate buffer, pH 6.8 at 4°. The column and the enzyme sample were equilibrated in the above buffer containing the appropriate substrate or constituent part of NAD^+.

Substrate or Constituent	Apparent Molecular Weight
0.2 mM Oxalacetic Acid	51,000
1.0 mM L-Malate	51,000
0.1 mM Adenine	35,000
0.1 mM Adenosine	53,000
0.1 mM AMP	67,000
2.0 mM NaCl	38,000
no addition	35,000

of dimer formation (4,5). Adenosine and AMP, component parts of NAD^+, were utilized in order to ascertain their effect upon the monomer-dimer equilibrium. As noted in Table I, adenine had little or no effect on the equilibrium while adenosine was found to shift the equilibrium partially towards the dimer form of the enzyme. The addition of AMP, however, facilitated almost complete reassociation of the enzyme into the dimeric form. Thus, the minimum component of NAD^+ that is apparently able to affect complete reassociation of malate dehydrogenase is AMP. These results are consistent with those observed in studies utilizing the analytical ultracentrifuge to investigate the pH-dependent dissociation of this enzyme at high enzyme concentrations (5).

In order to demonstrate that ionic strength was not a significant factor in the monomer-dimer equilibrium in the presence of substrates or cofactor, a sample of malate dehydrogenase at 0.1 μM was chromatographed in the presence of 2.0 mM NaCl (Table I). The finding of an apparent molecular weight of 38,000 indicates that ionic strength was not a contributing factor in the displacement of this equilibrium towards dimer by substrates.

Fluorescence polarization studies of malate dehydrogenase by Shore and Chakrabarti (3) performed at pH 8.0 in 50 mM Tris-acetate buffer at 25° have

also demonstrated a concentration-dependent dissociation of this enzyme. In the work by Shore and Chakrabarti (3) it was observed that while the presence of NAD^+ (10 mM) facilitated the reassociation of malate dehydrogenase under these conditions, the presence of 25 μM NADH possibly enhanced the degree of dissociation of the enzyme. Recent studies reported by this laboratory (4,5) have suggested that both NADH and NAD^+ acted to facilitate the reassociation of malate dehydrogenase under conditions of either low pH or low protein concentration.

In view of the reported differential effect of NADH upon this sytem we have reinvestigated the effects of this coenzyme under conditions similar to those used by Shore and Chakrabarti (3) (50 mM Tris-acetate buffer at pH 8.0 at 25° using an enzyme concentration of 0.1 μM). It was observed that 25 μM NADH was insufficient to effect reassociation of the enzyme. However, it was observed that 100 μM NADH will cause reassociation of malate dehydrogenase. This data thus suggests that the previous authors simply utilized insufficient concentrations of NADH to effect reassociation of this enzyme and hence were led to an erroneous conclusion that the two coenzymes act differently in the matter of displacement of the monomer-dimer equilibrium for malate dehydrogenase

The above results strongly suggest that conditions of pH and protein concentration must be considered in studies of mitochondrial malate dehydrogenase dealing either with substrate and coenzyme binding as they relate to structure-function relationships or with subunit interactions. In light of these findings it is obvious that conditions of protein concentration and pH utilized in the investigation of this enzyme have a profound effect upon the position of the monomer-dimer equilibrium.

Studies utilizing fluorescence enhancement titration techniques (6) and circular dichroism properties (7) to investigate the binding properties of NAD^+ and NADH to malate dehydrogenase have recently been performed. The conditions of pH and protein concentration utilized in those studies now appear to be conditions which favor the formation of monomers of this enzyme. Hence, an

investigation of the cooperativity of coenzyme binding using these conditions could only have led to the conclusion that the binding sites are independent. Work recently reported by this laboratory (11) reinvestigating the nature of coenzyme binding to mitochondrial malate dehydrogenase at enzyme concentrations and pH values favoring dimer formation have demonstrated that the binding of reduced coenzyme is indeed anticooperative.

Similar protein concentration and pH-dependent dissociations have been shown to exist in many multisubunit enzyme complexes. When a complex exhibits both a protein concentration and pH dependent dissociation it is important to consider both of these variables in experiments which require homogeneity of the quaternary structure. Failure to consider both variables can lead to discrepancies between data obtained at the same protein concentration but at different pH values. Thus in view of the considerable recent interest in studies dealing with molecular asymmetry in binding of substrates and cofactors to such complexes, investigators studying these aspects must be fully aware of the total effects of experimental conditions upon the enzyme under investigation.

ACKNOWLEDGEMENTS

This investigation was supported by Grant HL-12585 from the National Institutes of Health. (J.H.H.) Recipient of Career Development Award K4-HL-70-728. (C.T.H.) Partially supported during preliminary predoctoral training by Project 236 from the Bureau of Community Health Service, Health Services Training Branch, Health Services Administration, and by Grant HD-03110 from the National Institute of Child Health and Human Development, National Institutes of Health

REFERENCES

1. Cournil, I., Barba, J.-M., Verge, D., and Arrio-Dupont, M (1975) J. Biol. Chem. 250, 8564-8568.

2. Pauly, H.E., Pfleiderer, G. (1977) Biochemistry 16, 4599-4604.

3. Shore, J.D., and Chakrabarti, S.K. (1976) Biochemistry 15, 875-879.

4. Bleile, D.M., Shulz, R.A., Gregory, E.M., and Harrison, J.H. (1977) J. Biol. Chem. 252, 755-758.

5. Hodges, C.T., Wiggins, J.C., and Harrison, J.H. (1977) J. Biol. Chem. 252, 6038-6041.

6. Holbrook, J.J., Wolfe, R.G. (1972) Biochemistry 11, 2499-2502.

7. Eberhardt, N.L., Wolfe, R.G. (1975) J. Biol. Chem. 250, 2987-2992.

8. Gregory, E.M., Yost, F.J., Jr., Rohrbach, M.S., and Harrison, J.H. (1971) J. Biol. Chem. 246, 5491-5497.

9. Frieden, C., and Honegger, J., and Gilbert, H.R., (1978) J. Biol. Chem. 253, 816-820.

10. Davies, D.D., and Kun, E. (1975) Biochem. J. 66, 307-316.

11. Hodges, C.T., Wood, D.C., and Harrison, J.H., (1978) J. Biol. Chem. "in press".