The Effect of Inorganic Ions on the Structure and Function of Mitochondrial Malic Dehydrogenase from Bovine Heart Muscle*

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ABSTRACT: Mitochondrial malic dehydrogenase (M-MDH), isolated from bovine heart muscle, was reversibly dissociated in 0.2 M sodium citrate at pH 2.6. The molecular weight of the resultant subunits was approximately 17,000 or about one-fourth of the native enzyme. Reconstitution of MDH from its subunits occurred in high concentrations of either phosphate, arsenate or Tris, at neutral pH. Reversible dissociation of MDH in 4 M urea was also observed. Resultant subunits of molecular weight about 15,000 could be recombined under certain conditions of high ionic strength to regenerate the enzyme. MDH, when reacted with *p*-hydroxymercuribenzoate (PMB) was reversibly inactivated but did not appear to dissociate. Reactivation of PMB-treated MDH occurred under conditions similar to those needed to recombine the subunits formed upon acid or urea exposure. The possible role of inorganic ions in the reactivation and recombination processes is discussed.

Munkres (1965a,b) has reported the dissociation of *Neurospora* MDH[1] into four subunits in acid and urea. Chilson *et al.* (1965, 1966) have described the dissociation and inactivation of pig and chicken heart M-MDH in acid and guanidine hydrochloride solutions. These workers have been able to reactivate the enzyme after exposure to these two reagents. Murphey *et al.* (1967) recently reported the reversible dissociation of MDH from *Bacillus subtilis* and *Bacillus stearothermophilus*. Harrison (1963) has described the dissociation of bovine MDH with either 1,10-phenanthroline or with a polymeric substance present in preparations of lipoic acid.

The present paper deals with the reversible dissociation of beef heart M-MDH into subunits in acid and urea and the specific conditions required to reconstitute the enzyme from its subunits. These conditions will be shown to be different from those reported by the above workers for MDH isolated from other sources. In addition, it will be shown that the beef heart enzyme can be reversibly inactivated by PMB without apparent dissociation of the protein. The conditions required for the reactivation of dissociated MDH compared with nondissociated MDH will be discussed.

Experimental Section

Materials

Oxalacetic acid and l-malic acid were obtained from Calbiochem. PMB obtained from the Sigma Chemical Co. was recrystallized by the method of Boyer (1954). Recrystallized bovine serum albumin was obtained from the Pentax Corp., and NAD and NADH were from the Sigma Chemical Co. The cation-exchange resin, AG 50W-X8 (200–400 mesh), was purchased from Bio-Rad Laboratories and washed with 1 M KOH and 1 M HCl prior to use. Sephadex G-75 (medium) was obtained from Pharmacia, Uppsala, Sweden.

Methods

In all experiments, unless otherwise specified, MDH was equilibrated by dialysis with 0.001 M potassium phosphate at pH 7.4 prior to use. The enzyme assays were conducted by methods previously described (Siegel and Englard, 1961; Englard and Breiger, 1962; Siegel and Englard, 1962). Protein determinations were done by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

The diffusion experiments were performed in a Tiselius cell using a Perkin-Elmer Model 238 electrophoresis-diffusion instrument. Before each diffusion run the protein was equilibrated by dialysis with the appropriate medium.

Estimation of molecular weight on Sephadex G-75 was done by a slight modification of a method by Andrews (1964). A column (88 × 1.5 cm) was prepared and equilibrated with 0.001 M Tris (Cl^-) and 0.05 M KCl at pH 7.8. A standard curve was prepared with several proteins of known molecular weight and the procedure was carried out at 25°.

The sedimentation coefficients were determined using a Spinco Model E ultracentrifuge and a schlieren optical system. Inorganic phosphate was determined by the method of Fiske and Subbarow (1925).

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[1] Abbreviations used: M-MDH, mitochondrial malic dehydrogenase; PMB, *p*-hydroxymercuribenzoate; NAD, nicotinamide-adenine dinucleotide; NADH, reduced NAD; AMP, adenosine monophosphate.

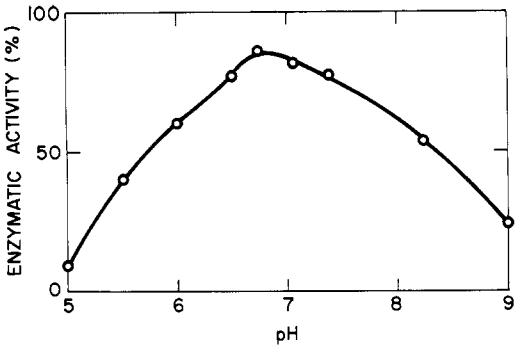

FIGURE 1: Reactivation of MDH as a function of pH after inactivation at pH 2.6. MDH (2.0 mg) was incubated in 2.0 ml of 0.2 M sodium citrate at pH 2.6 for 15 min at 0°. The enzyme was then diluted to 0.1 mg/ml in 1.0 M potassium phosphate with a suitable amount of potassium hydroxide in order to achieve the appropriate pH values. This mixture was incubated for 15 min at 25° and aliquots were removed for assay.

TABLE I: Effect of Various Ions on the Reactivation of MDH after Inactivation at pH 2.6.[a]

	% Enzymatic Act. Restored
No addition	6
Potassium phosphate	72
Sodium phosphate	70
Sodium pyrophosphate	12
AMP (0.1 M)	10
K_2SO_4	10
KCl	7
KNO_3	7
Sodium arsenate	68
Tris (SO_4^{2-})	70
β-Mercaptoethanol (0.1 M)	11
Sodium malate	6
Sodium oxalacetate	7
NAD (0.001 M)	8
NADH (0.001 M)	9

[a] MDH (2.0 mg) was incubated in 2.0 ml of 0.2 M sodium citrate at pH 2.6 for 15 min at 0°. The protein was then diluted to 0.1 mg/ml at pH 6.7 (adjusted with KOH) in 0.5 M (concentrations not 0.5 M also indicated) solutions of the salts listed below, and allowed to incubate for 15 min at 25°. Suitable aliquots were removed for assay.

Results

Purification of MDH from Bovine Heart Muscle. MDH was purified by a slight modification of a procedure previously described (Siegel, 1962). Ion-exchange chromatography was substituted for starch block electrophoresis in the final step of purification. After equilibration with 0.005 M potassium phosphate buffer by dialysis at 5°, the protein was applied to a Bio-Rad AG 50W-X8 (200–400 mesh) column (45 × 4.0 cm). The procedure consisted of washing the column with 0.005 M potassium phosphate (pH 7.0) in order to remove extraneous protein from the enzyme preparation. A gradient of increasing ionic strength was then established at pH 7.0 with potassium phosphate, resulting in elution of MDH in about 0.02 M phosphate. The purified enzyme (diluted in 0.1 M potassium phosphate (pH 7.4) prior to assay) catalyzed the oxidation of 350 μmoles of potassium malate/min per mg of protein by NAD in 0.1 M glycine buffer at pH 10.0. Purified M-MDH was homogeneous in the analytical ultracentrifuge and also by moving-boundary electrophoresis when tested over the pH range 5.5–8.8.

Dissociation of MDH in 0.2 M Sodium Citrate (pH 2.6). The incubation of MDH (1.0–5.0 mg/ml) in 0.2 M sodium citrate (pH 2.6) caused complete loss of enzymatic activity within 15 min at 0°. Neutralization of this incubation mixture with KOH (to a final pH of 6.7) resulted in a 6% restoration of enzymatic activity (Table I). However, neutralization with KOH in the presence of 0.5 M concentrations of phosphate, arsenate, or Tris (sulfate or chloride) caused about 70% restoration of enzymatic activity. No other substance tested, including the substrates for MDH, caused a significant restoration of enzymatic activity. When MDH was incubated in 0.5 M potassium phosphate (pH 2.6) at 0°, a rapid loss of enzymatic activity was observed, indicating that phosphate did not protect the enzyme against this low pH.

Reactivation of the enzyme by potassium phosphate after an incubation at pH 2.6 was studied as a function of pH (Figure 1). Noticeable restoration of enzymatic activity was observed from pH 5.0 to 9.0 with maximum reactivation at pH 6.7. Reactivation of MDH as a function of phosphate concentration at pH 6.7 is shown in Figure 2. It can be seen that rather high concentrations of phosphate are required for maximal restoration of activity. In other experiments, it was found that 0.001 M NADH enhanced the reactivation of MDH by phosphate approximately 10–15% over the pH range 5.0–7.0. Neither NAD, malate, nor oxalacetate could substitute for NADH in this respect.

During the incubation of MDH in 0.2 M sodium citrate (pH 2.6) at 0°, aliquots were withdrawn at various time intervals, neutralized in 1 M potassium phosphate at pH 6.7, and subjected to enzymatic assay (Figure 3). The ability to reactivate the enzyme decreased with time. At the end of 3 hr at pH 2.6, 0°, only 3% of the enzymatic activity could be restored. When MDH was incubated at pH 2.6 at 25°, less restoration of enzymatic activity was achieved.

Sedimentation Studies of MDH in 0.2 M Citrate at pH 2.6, 5°. Incubation of MDH in 0.2 M sodium citrate at pH 2.6 at 5° caused dissociation of the enzyme into homogeneous material with an $s_{20,w}$ of 1.9 (Table II). This sedimentation coefficient remained constant over a

TABLE II: Sedimentation and Diffusion of MDH in 0.2 M Sodium Citrate (pH 2.6).[a]

Expt	Conditions	$s_{20,w}$ (S)	$D_{20,w}$
1	MDH in 0.1 M potassium phosphate (pH 7.4) at 5°	4.3	6.5
2	MDH in 0.2 M citrate for 30 min at 5°	1.9	
3	MDH in 0.2 M citrate for 60 min at 5°	1.9	
4	MDH in 0.2 M citrate for 24 hr at 5°	1.9	9.5
5	MDH in 0.2 M citrate for 30 min at 25°	6.5	
6	MDH in 0.2 M citrate for 60 min at 25°	6.4	
7	MDH in 0.2 M citrate for 60 min at 5°, neutralized with 0.5 M potassium phosphate at pH 6.7, then dialyzed against 0.1 M potassium phosphate (pH 6.7) at 5° for 15 hr	4.2	

[a] The protein concentration in expt 1–6 was 5.0 mg/ml. In expt 7, the protein concentration was 8.0 mg/ml during the incubation at pH 2.6, and 3.1 mg/ml after neutralization and dialysis. The initial photograph of the sedimenting protein was taken at the end of the incubation periods specified.

period of 24 hr, suggesting that no further decrease in molecular weight occurred after the initial dissociation. A diffusion coefficient ($D_{20,w}$) of 9.5 was obtained for the dissociated protein under identical conditions of incubation. From these data a molecular weight of approximately 17,000 was calculated for the subunits. Evidence that the dissociation of MDH into subunits was reversible was obtained by neutralization of the acidified enzyme (after 60 min at pH 2.6, 5°) with KOH in 0.5 M potassium phosphate (pH 6.7). A precipitate formed during neutralization (consisting of about 50% of the total protein) which was removed by centrifugation and found to have no enzymatic activity. The supernatant solution was equilibrated by dialysis with 0.1 M potassium phosphate at pH 6.7 and examined in the ultracentrifuge. An $s_{20,w}$ of 4.2 was obtained for this material which was almost identical with that of the native enzyme. In addition the supernatant solution now contained about 40% of the total enzymatic activity, and 85% of the specific activity of pure MDH. When this solution was chromatographed on a Sephadex G-75 column (see Methods) a molecular weight of about 65,000 was found, identical with that of the native enzyme. In addition, when native MDH and reconstituted MDH were combined, and chromatographed on Sephadex G-75, only one peak was observed.

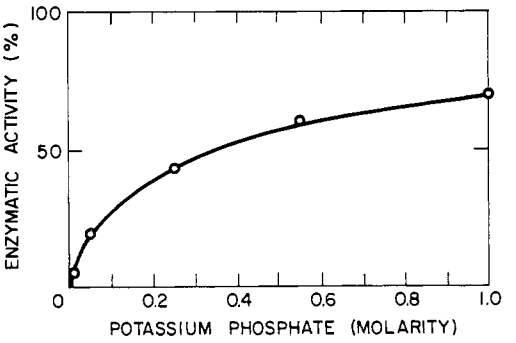

FIGURE 2: Reactivation of MDH as a function of phosphate concentration after inactivation at pH 2.6. MDH (2.0 mg) was incubated in 2.0 ml of 0.2 M sodium citrate at pH 2.6 for 30 min at 0°. The enzyme was then diluted to 0.1 mg/ml at pH 6.7 in varying concentrations of potassium phosphate. This mixture was allowed to stand 15 min at 25° and suitable aliquots were removed for assay.

When MDH was incubated at pH 2.6 at 5° for 60 min, neutralized with KOH alone to pH 6.7, and chromatographed on a Sephadex G-75 column, the resulting material had a molecular weight of about 90,000 and was enzymatically inactive. Apparently, the absence of high phosphate concentration during neutralization resulted in recombination of subunits into a protein of higher molecular weight than native MDH. Furthermore, the incubation of MDH at pH 2.6 at 25° gave rise to a component with a $s_{20,w}$ of 6.5 (Table II) which also had a molecular weight of approximately 90,000, as judged by chromatography on Sephadex G-75. This was also enzymatically inactive.

Dissociation of MDH in 4 M Urea. The incubation of MDH (1.0–5.0 mg/ml) in 4 M urea buffered with 0.0001

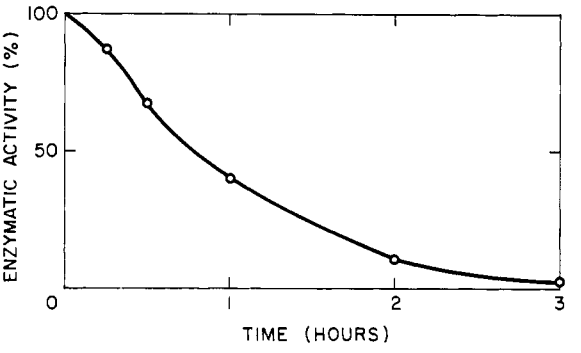

FIGURE 3: Reactivation of MDH as a function of incubation time at pH 2.6. Enzyme (10.0 mg) was incubated in 2 ml of 0.2 M sodium citrate at pH 2.6, 0°. Aliquots were removed at various time intervals, diluted to 0.5 mg/ml (in protein) in 1 M potassium phosphate at pH 6.7, and allowed to stand for 15 min at 25°. Suitable aliquots were removed for assay.

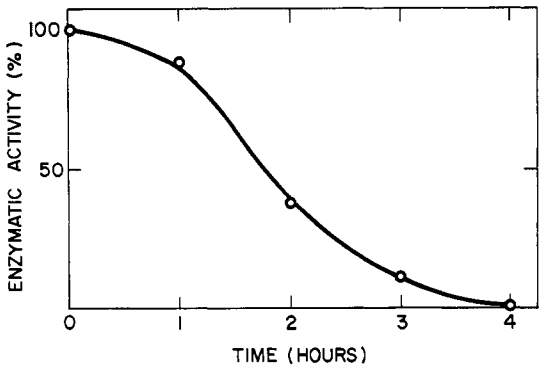

FIGURE 4: Reactivation of MDH as a function of incubation time in 4 M urea buffered with 0.001 M potassium phosphate at pH 7.4. At various time intervals aliquots were removed, diluted to 0.5 mg/ml in 1 M potassium phosphate at pH 8.0, and allowed to stand 15 min at 25°. Suitable aliquots were subjected to enzymatic assay.

M potassium phosphate at pH 7.4 caused complete loss of enzymatic activity within 30 min at either 0 or 25°. The addition of either sodium or potassium phosphate, sodium arsenate, or Tris (sulfate or chloride) to a final concentration of 0.5 M protected the enzyme completely in 4 M urea for 30 min at 0°. Solutions (0.5 M) of potassium sulfate, nitrate, and chloride were 58, 38, and 41%, respectively, as effective as phosphate. β-Mercaptoethanol (0.1 M) was 48% as effective as phosphate, and 0.001 M solutions of NADH and NAD were 70 and 30%, respectively, as effective as phosphate in maintaining MDH activity in the presence of 4 M urea at pH 7.4.

Attempts were made to reactivate MDH which had been incubated in 4 M urea for 30 min at 0° (Table III). Dilution of the enzyme in 1 M solutions of phosphate, arsenate, or Tris caused appreciable restoration of enzymatic activity. Unlike MDH incubated at pH 2.6, the enzyme in 4 M urea was partially reactivated by other ions, in addition to 0.001 M solutions of either NAD or NADH and 0.1 M β-mercaptoethanol. Thus, a broader specificity for reactivation was observed. In addition, whereas phosphate did not protect the enzyme against loss of activity in 0.2 M citrate at pH 2.6, it did protect the enzyme in 4 M urea. Reactivation of MDH which had been incubated in urea required high concentrations of phosphate, with optimal restoration of enzymatic activity in 1 M phosphate, similar to that observed for the reactivation of acidified MDH. Reactivation of "urea-treated MDH" was optimal at pH 8.2, with at least 70% activity restored over the pH range 7.0–9.4 and 25% reactivation as low as pH 6.2.

During the incubation of MDH in 4 M urea, aliquots were removed, diluted in 1 M potassium phosphate at pH 8.0, and subjected to enzymatic assay. The results of such an experiment are shown in Figure 4. Approximately 90% of the enzymatic activity was restored after 60 min in 4 M urea; however, the ability to reactivate the enzyme decreased with prolonged incubation in urea.

TABLE III: Reactivation of MDH after Inactivation in 4 M Urea.[a]

Addition[b]	% Enzymatic Act. Restored
None (H_2O)	0
Potassium phosphate (1 M)	85
Sodium arsenate (1 M)	80
Tris (SO_4^{2-}) (1 M)	90
Tris (Cl^-) (1 M)	87
KCl (1 M)	24
NADH (0.001 M)	52
NAD (0.001 M)	40
β-Mercaptoethanol (0.1 M)	28
K_2SO_4 (1 M)	67

[a] Enzyme (2.0 mg) was incubated in 2.0 ml of 4 M urea buffered with 0.0001 M potassium phosphate at pH 7.4 for 60 min at 0°, and then diluted to 0.1 mg/ml with the salts indicated at the specified concentrations. Each mixture was allowed to stand for 15 min at 25° prior to assay. [b] The final pH was adjusted to 7.4 with dilute KOH when necessary.

Sedimentation Studies of MDH in 4 M Urea. When MDH was incubated at either 5 or 25° in 4 M urea and examined in the ultracentrifuge, a single peak was observed with an $s_{20,w}$ of 1.7. This value remained constant at 5° over a period of 24 hr (Table IV). When the sedimentation experiments were performed in 4 M urea in the presence of 0.5 M potassium phosphate at pH 7.4, a single peak was observed with an $s_{20,w}$ of 4.4, almost identical with that of the native enzyme. This was not unexpected since phosphate prevented loss of enzymatic activity in 4 M urea and apparently prevented dissociation. The material with an $s_{20,w}$ of 1.7 (incubated in 4 M urea at 5° for 60 min) was dialyzed against 0.5 M potassium phosphate at pH 7.4 and then equilibrated by dialysis with 0.1 M potassium phosphate at pH 7.4. A slight precipitate which formed during dialysis was removed by centrifugation and the supernatant solution examined in the ultracentrifuge. This material had an $s_{20,w}$ of 4.3, 54% of the total enzymatic activity, and 82% of the original specific activity. Chromatography of this protein solution on a Sephadex G-75 column revealed a molecular weight of about 67,000, very similar to the molecular weight of the native enzyme. The diffusion coefficient of the enzyme in 4 M urea was 9.5. The molecular weight of the subunits in urea was calculated to be about 15,000.

Attempts to determine the molecular weight of the subunits independently, on a Sephadex G-75 column were not successful. Removal of urea from the enzyme resulted in spontaneous aggregation of these peptides to a form having a molecular weight of about 95,000. It was also not possible to obtain a reliable molecular weight on Sephadex G-75 in the presence of urea due to

TABLE IV: Sedimentation and Diffusion of MDH in 4 M Urea.[a]

Expt	Conditions	$s_{20,w}$ (S)	$D_{20,w}$
1	MDH in 0.1 M potassium phosphate (pH 7.4)	4.3	6.5
2	MDH in 4 M urea, 60 min at 25°	1.7	
3	MDH in 4 M urea, 30 min at 25°	1.7	
4	MDH in 4 M urea, 3 hr at 25°	1.8	
5	MDH in 4 M urea, 60 min at 5°	1.7	
6	MDH in 4 M urea, 24 hr at 5°	1.7	9.2
7	MDH in 4 M urea, 60 min at 0°, then adjusted to 0.5 M in potassium phosphate (pH 7.4) and dialzyed for 12 hr against 0.1 M potassium phosphate (pH 7.4)	4.3	
8	MDH in 4 M urea plus 0.3 M potassium phosphate (pH 7.4) for 60 min at 25°	4.4	

[a] Unless otherwise specified, the urea solutions were buffered with 0.001 M potassium phosphate (pH 7.4). The protein concentration in each experiment was 5.0 mg/ml. The incubation time in each experiment except expt 7 represents the time that the initial photograph was taken of the sedimenting protein.

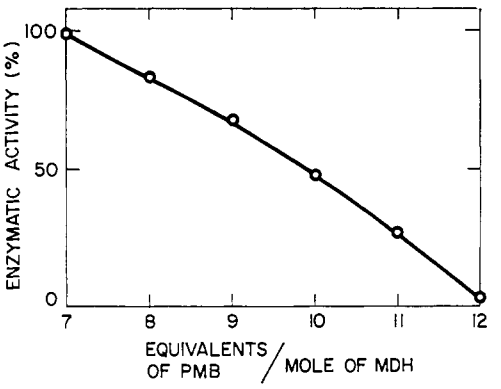

FIGURE 5: Reaction of PMB with the sulfhydryl groups of MDH. MDH (0.62 mg, 0.01 μmole) was incubated in separate tubes with different amounts of PMB in 0.001 M potassium phosphate at pH 7.4 for 2 hr at 25°; total volume, 3.0 ml. Estimation of mercaptide formation at 250 mμ indicated that the reactions were complete (Boyer, 1954). Aliquots were removed for enzymatic assay.

the problem of obtaining a standard curve with known proteins in urea solutions.

The Reaction of MDH with PMB. It was previously shown that beef heart MDH was rapidly inactivated by PMB (Siegel and Englard, 1962). It was, therefore, of interest to see if this reagent could cause dissociation of the enzyme into subunits. The enzyme, which contains 12 sulfhydryl groups/molecule (Siegel and Englard, 1962), was treated in a stepwise manner with PMS and these results are shown in Figure 5. Approximately 6 equiv of PMB reacted with MDH, causing no loss of enzymatic activity. The further addition of PMB resulted in a gradual decrease in activity until all 12 sulfhydryl groups had reacted. At this point no activity remained.

When the enzyme was incubated with a 60-fold excess of PMB at 0°, complete loss of enzymatic activity occurred within 15 min. The presence of 1 M potassium phosphate partially protected the enzyme against loss of activity by PMB with 50% enzymatic activity remaining after 30 min, 20% after 60 min, and 5% after 90 min. Tris (sulfate or chloride) could replace phosphate on an equimolar basis. In the presence of 1 M potassium sulfate, 25% enzymatic activity remained after 30 min, and only 5% after 60 min. Other ions such as chloride and nitrate were not significantly effective in protecting MDH against PMB.

After MDH reacted with PMB (60 equiv) for 30 min at 0°, aliquots were removed and diluted in 0.1 M β-mercaptoethanol in addition to other salts shown in Table V. The presence of Tris (Cl^-) or phosphate caused appreciable reactivation of the enzyme. Sulfate, chloride, and nitrate were less effective, as was β-mercaptoethanol itself. However, β-mercaptoethanol was required for restoration of enzymatic activity, since phosphate or

TABLE V: Reactivation of MDH after Inactivation by PMB.

Addition	% Enzymatic Act. Restored
None	18
K_2PO_4	89
Tris (Cl^-)	85
K_2SO_4	51
KCl	21
KNO_3	20

[a] MDH (2.0 mg) was incubated with PMB (60 equiv/mole of enzyme) in 2.0 ml of 0.001 M potassium phosphate at pH 7.4 for 30 min at 0°. The enzyme was then diluted to 0.1 mg/ml in 0.1 M β-mercaptoethanol in addition to 1 M solutions of the salts below at pH 9.0 (adjusted with KOH). These mixtures were allowed to stand for 15 min at 25° and aliquots were removed for assay.

Tris alone were almost completely ineffective. The phosphate concentration required for reactivation of MDH was approximately the same as that required for restoration of activity after incubations in either 4 M urea at pH 7.4 or 0.2 M citrate at pH 2.6. Maximum reactivation occurred in 1 M potassium phosphate. Restoration of MDH activity after treating the enzyme with PMB occurred over a broad pH range (5.0–10.0) with a slight maximum at pH 9.2. Prolonged incubation of MDH in PMB solution (3 hr at 0°) caused irreversible inactivation.

Sedimentation Studies on MDH after Reaction with PMB. When MDH was treated with a 60-fold excess of PMB at either 5 or 25° and then examined in the ultracentrifuge, a single peak was observed with an $s_{20,w}$ of 4.1 and 4.0, respectively (Table VI). Further incubation with PMB at 25° caused the emergence of a second peak with an $s_{20,w}$ of 8.1. Finally, prolonged incubation caused extensive precipitation which made it impossible to determine a diffusion coefficient for the protein in the presence of PMB. For this reason it was also not possible to obtain an independent confirmation of the molecular weight using Sephadex G-75. The sedimentation data suggest, however, that MDH does not dissociate when treated with PMB. The slight decrease in sedimentation coefficient compared with native MDH may be due to conformation changes in the molecule.

TABLE VI: Sedimentation of MDH after Treating the Enzyme with PMB.[a]

Expt	Conditions	$s_{20,w}$ (S)
1	Native MDH	4.3
2	Native MDH plus 60 equiv of PMB at 5° for 3 hr	4.1
3	Native MDH plus PMB for 1 hr at 25°	4.0
4	Native MDH plus PMB for 3 hr at 25°	4.0, 8.1[b]

[a] MDH (4.0 mg) was incubated with PMB (60 equiv/mole of protein) for the designated time periods in 0.001 M potassium phosphate (pH 7.4). Total volume, 1.0 ml. [b] Two peaks were observed. About 90% of the material had an $s_{20,w}$ of 4.0 S and approximately 10% had a value of 8.1 S.

Discussion

The experiments described in this paper strongly suggest that MDH from bovine heart muscle can reversibly dissociate into four subunits. These findings are in accord with the observations of Munkres (1965a,b) on MDH isolated from *Neurospora*. Similarly, Chilson *et al.* (1965, 1966) have also described the dissociation of MDH isolated from chicken heart and pig heart into subunits. Also, the reversible dissociation of MDH from *B. subtilis* and *B. stearothermophilus* has recently been reported (Murphey *et al.*, 1967).

When the beef heart enzyme was incubated in 0.2 M citrate (pH 2.6) at 5°, a single peak was observed in the ultracentrifuge with an $s_{20,w}$ of 1.9 and a molecular weight of about 17,000. Neutralization of this enzymatically inactive material with a high concentration of phosphate, arsenate, or Tris caused the appearance of a substance with an $s_{20,w}$ of 4.2 and a molecular weight of 65,000, as judged by chromatography on Sephadex G-75. This reconstituted protein had regained appreciable enzymatic activity. Neutralization with potassium hydroxide alone resulted in the appearance of a substance with a molecular weight of about 90,000 and no enzymatic activity. In other experiments, MDH, dissolved in 0.01 M sodium citrate, 0.05 M NaCl, 0.001 M EDTA, and 0.001 M β-mercaptoethanol at pH 6.8, was adjusted to pH 2.8 with citric acid and incubated at this pH for 5 min until no enzymatic activity remained. Neutralization of this solution to pH 6.8 caused no appreciable reactivation, indicating that this enzyme differed from *Neurospora* MDH inasmuch as the latter could be reactivated under these conditions (Munkres, 1965a,b). Also, beef heart MDH, after acidification at pH 2.6, could not be reactivated in 0.1 M citrate containing 0.1 M β-mercaptoethanol at pH 7.0. These conditions were effective in the reactivation of pig and chicken heart mitochondrial MDH after acidification (Chilson *et al.*, 1966). Incubation of MDH at pH 2.6 at 25° caused the rapid appearance of a substance with an $s_{20,w}$ of 6.5 and a molecular weight of approximately 90,000. This material had no enzymatic activity and could not be reactivated. Prolonged incubation of MDH at pH 2.6 at 5° did not cause a further decrease in sedimentation coefficient, although the ability to restore enzymatic activity decreased considerably with time. These observations suggested that structural changes occurred in the protein with no further decrease in molecular weight. Thus, the structure of the subunits became altered to the extent where they could no longer combine to reform the native enzyme.

The possible role of inorganic ions as a bridge linking together peptide subunits was examined. During neutralization of acidified MDH, radioactive [^{32}P]phosphate was added in order to determine if nondialyzable phosphate could be incorporated into the reconstituted protein. The results, however, did not support this hypothesis since there was no significant incorporation of [^{32}P]-phosphate during reconstitution of the enzyme.

Further evidence for the existence of malic dehydrogenase as four peptide chains was provided by dissociation of the enzyme in 4 M urea. A rapid loss of enzymatic activity in urea solution was prevented by the presence of high concentrations of various salts. Phosphate, arsenate, and Tris protected the enzyme maximally, whereas other salts in addition to the coenzymes NAD and NADH protected the enzyme to a lesser extent. Seguin (1966) has observed partial protection of pig heart MDH by phosphate in 4 M urea. Examination of

MDH in 4 M urea revealed a substance with a molecular weight of about 15,000, as determined by sedimentation diffusion experiments. Reactivation of urea-dissociated MDH occurred at high salt concentration, with maximum restoration of activity in solutions of phosphate, arsenate, or Tris. Reconstitution of MDH after dissociation in urea gave rise to a protein identical in molecular weight with the native enzyme. Prolonged incubation in 4 M urea resulted in complete inability to reactivate the enzyme, although no further decrease in sedimentation coefficient was observed during this period. These findings were similar to those observed during the dissociation of MDH at pH 2.6 and suggest that irreversible changes in the three-dimensional structure of the subunits eventually take place.

Since PMB was found to completely inhibit MDH (Siegel and Englard, 1962) it was of interest to see if this reagent would also cause dissociation of the enzyme. Incubation of MDH with an excess of PMB caused a rapid and complete loss of enzymatic activity. Partial protection of the enzyme against this reagent was afforded by high concentrations of phosphate, arsenate, or Tris. Other salts tested protected the enzyme to a much lesser extent. After incubation with PMB, maximum reactivation of MDH occurred with the above three salts in conjunction with β-mercaptoethanol. No restoration of enzymatic activity was observed with phosphate, arsenate, or Tris in the absence of β-mercaptoethanol, which itself was only slightly effective. Furthermore, the concentration of phosphate required to reactivate PMB-treated MDH was comparable to that required for reconstitution of the enzyme after exposure to urea and acid. The product resulting from the complete reaction of MDH with PMB was examined in the ultracentrifuge. A single peak with an $s_{20,w}$ of 4.1 was observed, indicating that no dissociation occurred. Thus, reactivation of this protein appeared to involve structural changes in the intact molecule.

It is, therefore, apparent that the reconstitution of MDH from its subunits occurs under similar conditions to those required for the reactivation of nondissociated, inactive MDH. Thus, it is suggested that the role of high salt concentration in the reconstitution of the enzyme from its subunits involves either: (1) inducement of conformational changes in the subunit structure followed by spontaneous recombination to regenerate the native enzyme, or (2) a twofold effect involving inducement of structural changes in the subunits in addition to a direct participation of the inorganic ions during the recombination process. The preferential requirement for certain ions during recombination of subunits, particularly after dissociation of the enzyme at pH 2.6, implies a more intimate role for these ions other than providing a medium of high ionic strength. Studies are now in progress to provide further evidence as to the number of subunits contained in the native enzyme, the chemical nature of these subunits, and the structural changes associated with the inactivation and reactivation processes.

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