Beef Heart Malic Dehydrogenases

VII. Reactivity of Sulfhydryl Groups and Conformation of the Supernatant Enzyme*

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SUMMARY

Only 3 of the 6 sulfhydryl groups of native bovine heart supernatant malate dehydrogenase (S-MDH) react with p-mercuribenzoate (PMB) with no loss of enzymatic activity. In addition, prolonged incubation of S-MDH in 2.6 M urea solutions does not significantly alter the catalytic properties of the enzyme. In 2.6 M urea, however, all 6 sulfhydryl groups of the enzyme react with PMB, with attendant losses in catalytic activity. Under these conditions the rate of inactivation, although considerably slower than the rate of mercaptide formation, closely parallels the rate of change in the optical rotatory dispersion properties of the enzyme.

Native S-MDH exhibits an optical rotatory dispersion curve with a distinct shoulder in the 280 to 290 nm spectral region and a trough at 233 nm. Changes in these properties and other optical rotatory dispersion parameters were used to index conformational changes of S-MDH accompanying the various treatments. Native S-MDH and fully active S-MDH \( \cdot (\text{PMB})_3 \) exhibit identical optical rotatory dispersion curves. S-MDH in 2.6 M urea also exhibits an optical rotatory dispersion curve similar to that of the native enzyme. S-MDH \( \cdot (\text{PMB})_3 \) and S-MDH \( \cdot (\text{PMB})_3 \) in 2.6 M urea, however, have significantly different optical rotatory dispersion properties, including a disappearance of the 280 nm shoulder, decreases in \([m']_{280}\) values, and large decreases in \(-b_0\), \(a_0\), and \(b_0\) values. Since the rate of change in these optical rotatory dispersion properties closely paralleled the rate of loss of catalytic activity but not the rate of mercaptide formation, the results were interpreted as indicating that the loss in activity is related to the changes in protein conformation rather than to the actual blocking of sulfhydryl groups.

Extensive studies have been carried out in an attempt to define the role of sulfhydryl groups in the catalytic function of enzymes (2-4). For some enzymes, evidence has been presented for the direct participation of specific sulfhydryl groups in the catalytic process (5-9). In other enzymes, certain sulfhydryl groups have been implicated in the binding of coenzymes or substrates (10, 11) or have been shown to participate in the maintenance of the protein conformation essential for catalytic activity (12-15). In addition to the essential or nonessential nature of sulfhydryl groups in enzyme activity, thiol groups of proteins in the native state generally exhibit varying degrees of reactivity. Denaturation usually leads to exposure of newly reactive sulfhydryl groups, indicating that accessibility in the native state depends on their environment within the protein.

The two malate dehydrogenases (l-malate:NAD oxidoreductase, EC 1.1.37) from bovine heart, identified as being supernatant and mitochondrial in origin, have previously been compared and shown to differ in kinetic and physical properties and in amino acid composition (1, 16-19). Other than differences in content of particular amino acids, the outstanding distinction between the two enzymes is their interaction with and sensitivity to p-mercuribenzoate. M-MDH has 12 half-cystine residues as contrasted with 6 in S-MDH; however, neither enzyme contains disulfide bridges. All of the sulfhydryl groups of undenatured M-MDH can be titrated with PMB, although reaction occurs slowly, while only 3 of the 6 sulfhydryl groups of native S-MDH can be titrated even in the presence of excess PMB. The full complement of S-MDH thiol groups reacts after exposure of the enzyme to 6 M urea. A further difference is that titration of M-MDH with PMB results in the gradual loss of enzymatic activity after addition of only 3 equivalents of reagent, whereas no loss of activity of S-MDH occurs even after half its sulfhydryl groups have been titrated. In order to assess the role of the various sulfhydryl groups in bovine heart malate dehydrogenases, we have now examined the effects of modification of experimental conditions on the catalytic activity, on the reactivity of the sulfhydryl groups, and on the over-all three-dimensional structure of these enzymes.

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† Senior Research Fellow of the New York Heart Association.

‡ The abbreviations used are: M-MDH, mitochondrial malate dehydrogenase; S-MDH, supernatant malate dehydrogenase; PMB, p-mercuribenzoate.
**Modified preparation of bovine heart S-MDH**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity</th>
<th>Total proteins</th>
<th>Specific activity</th>
<th>Yield</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
<td>mg</td>
<td>units/mg</td>
<td>%</td>
</tr>
<tr>
<td>1. Crude extract...</td>
<td>228,599</td>
<td>22,480</td>
<td>10.2</td>
<td></td>
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<tr>
<td>2. First ammonium sulfate</td>
<td>199,013</td>
<td>6,964</td>
<td>28.6</td>
<td>87.1</td>
</tr>
<tr>
<td>fractionation...</td>
<td></td>
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<tr>
<td>3. Combined CM- and DEAE-</td>
<td>143,328</td>
<td>561.6</td>
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<td>133,978</td>
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<td>fractionation...</td>
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<td>84,029</td>
<td>124.4</td>
<td>675.5</td>
<td>36.8</td>
</tr>
</tbody>
</table>

**EXPERIMENTAL PROCEDURE**

**Reagents**

DPNH and oxaloacetic acid were obtained from Boehringer Mannheim. p-Mercuribenzoate (sodium salt) and mercapto-ethanol were purchased from Fluka AG Chemische Fabrik, Switzerland. Reduced glutathione and dithiothreitol were products of Calbiochem. "Ultra-pure" urea and enzyme grade ethanol were purchased from Fluka AG Chemische Fabrik, Switzerland.

**Preparation of Bovine Heart S-MDH**

Bovine heart S-MDH was purified and crystallized by a modification of the procedure of England and Breger (17). All steps were carried out at 2-4°C, and the ammonium sulfate saturations were calculated for 0°C.

**Steps 1 and 2**—The crude extract derived from 550 g of ground bovine heart muscle and the first ammonium sulfate fraction are prepared according to the original procedure (17). This latter fraction is now routinely collected by passing the suspension through a KSB-R Servall 8-tube continuous flow system at a rate of 30 to 40 ml per min at a rotor speed of 12,000 rpm. To reduce excessive foaming, the continuous flow apparatus is adapted with a modified (KSB-2086) high speed lift assembly. The 40 to 82% saturated ammonium sulfate residue is dissolved in 120 to 150 ml of 0.02 M potassium phosphate buffer (pH 6.9) containing 0.001 M EDTA and is dialyzed against this buffer for 36 to 48 hours; several changes of dialyzing medium are made. The dialyzed solution (320 to 370 ml) is clarified by centrifugation at 32,000 x g for 75 to 90 min.

**Step 3**—CM Sephadex (C 50, medium) and DEAE Sephadex (A-50, coarse) are suspended in 20 volumes of 0.2 M potassium phosphate buffer (pH 6.9) containing 0.001 M EDTA, stirred mechanically for 1 hour, and allowed to swell overnight. The ion exchange Sephadex gels are then washed successively with potassium phosphate buffers (pH 6.9) of decreasing concentration and the fine particles which do not settle within 20 min are removed by decantation. The gels are finally equilibrated with 0.02 M potassium phosphate buffer (pH 6.9) containing 0.001 M EDTA. A slurry of equilibrated carboxymethyl Sephadex is poured into a 4-cm internal diameter column (Chromaflex Chromatographic Apparatus, Kontes Glass Company, Vineland, New Jersey) and the gel is permitted to pack by gravity to a column height of 40 cm. In a similar manner, with the use of a K25/45 Sephadex laboratory column (Pharmacia Fine Chemicals, Inc.), a column, 2.5 x 40 cm, of the above equilibrated DEAE-Sephadex is prepared. The two columns are connected in series, so that the effluent emerging from the larger CM-Sephadex column flows directly into the top of the DEAE-Sephadex column. By inserting a single speed 1 rpm peristaltic type infusion pump (Micro-Flow tubing pump, model 7119-1, Cole-Parmer Instrument and Equipment Company, Chicago) between the two columns and using Tygon tubing with internal diameter 3/8 inch and external diameter 3/4 inch, a constant flow rate of 52 to 54 ml per hour is maintained for the combined Sephadex cation and anion chromatography system.

The dialyzed solution from Step 2 is applied to the top of the CM-Sephadex column and this is followed by passage of an equal volume of 0.02 M potassium phosphate buffer (pH 6.9) containing 0.001 M EDTA through the gel bed. Essentially all of the malate dehydrogenase of mitochondrial origin and the intensely colored red pigment are retained on the cation exchange Sephadex gel, and at this point S-MDH activity can no longer be detected in the eluate emerging from this column. The CM-Sephadex column is now disconnected from the combined chromatography system and the DEAE-Sephadex column is washed with an additional 500 to 600 ml of the same buffer; the flow rate is maintained with a constant delivery infusion pump. The column is next connected to a mixing flask containing 200 ml of 0.02 M potassium phosphate buffer (pH 6.9) in 0.001 M EDTA, which in turn is connected to a reservoir containing 0.1 M potassium phosphate buffer (pH 6.9) in 0.001 M EDTA. The mixing flask contains a rapidly spinning magnetic bar, and in this manner the concentration of potassium phosphate in solution entering the DEAE-Sephadex gel is increased gradually and uniformly. Fractions of approximately 10 ml are collected with an automatic fraction collector. After the gradient is started, malate dehydrogenase activity begins to emerge at an effluent volume of approximately 200 ml at a concentration of about 0.06 M potassium phosphate. The peak fractions with an over-all average increased specific activity of approximately 10-fold are pooled and fractionated as described below.

**Step 4**—The combined fraction (90 to 120 ml) obtained in Step 3 is brought to 55% saturation with salt by the slow addition of solid ammonium sulfate (38.5 g/100 ml). The mixture is stirred for 45 min and then centrifuged for 1 hour at 32,000 x g, and the inactive residue is discarded. Ammonium sulfate (17.7 g/100 ml of initial solution) is added gradually to the clear supernatant to 80% saturation and the mixture is stirred for 1 hour. The precipitate is collected by centrifugation at 32,000 x g for 1 hour and dissolved in a minimum volume of 0.05 M potassium phosphate (pH 6.9) containing 0.001 M EDTA. If necessary, the solution may be reduced in volume by means of pressure dialysis under nitrogen, and the enzyme crystallized from a 4.5 to 5.5% protein solution by following the details described in Step 6 of the original procedure (17).

Substitution of column chromatography on CM- and DEAE-Sephadex for several tedious steps of the original procedure (17) has conveniently shortened and simplified the method of purification of bovine heart S-MDH. The over-all yield of enzyme has been significantly increased, and preparations with specific activities approximately equal to those previously reported have been consistently obtained. The enzyme sediments as a single component on ultracentrifugation and migrates as a single band.

**TABLE I**

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Assay of S-MDH and Definition of Unit of Activity

Assays were carried out in cuvettes of 10-mm light path maintained at 28-30°C and containing, in a total volume of 3.0 ml, 150 µmoles of Tris (pH 7.6), 15 µmoles of EDTA, 0.38 µmole of freshly dissolved uncatalyzed oxaloacetic acid, and 0.45 µmole of DPNH. Reactions were initiated by addition of 10 µl of the various enzyme fractions or incubation mixtures diluted appropriately with 0.1 M potassium phosphate buffer, pH 7.4. Absorbance at 360 nm was continuously recorded with a Gilford model 2000 multiple sample absorbance recorder. A linear relationship was generally maintained for the first 5 to 6 min, provided the changes in absorbance did not exceed 0.035 per min.

One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 µmole of DPNH per min under the conditions of assay specified above. Specific activity is the number of units of enzyme activity per mg of protein; protein concentrations were determined by the method of Lowry et al. (21) with crystalline serum albumin as a standard.

Reaction of Sulfhydryl Groups with PMB

Sulfhydryl groups of S-MDH in the absence or presence of 2.6 M or 6 M urea were determined by the spectrophotometric titration procedure of Boyer (22). The concentrations of the stock solutions of PMB, prepared as previously described (18) were determined spectrophotometrically with the use of a molar absorbance coefficient at 232 nm of 1.69 x 10^4 (22). The mercurial solution was added stepwise to cuvettes containing known quantities of enzyme, and each addition of reagent generally represented a 1 mM equivalent of PMB per mole of enzyme present. The increments of absorbance of 255 nm due to mercaptide formation after each successive addition of reagent were measured in a Zeiss model PMQ II spectrophotometer. To ensure complete interaction, 10 min were allowed to elapse between the successive additions of PMB. Absorbance changes at 255 nm for PMB alone in each of the experiments were determined under identical conditions in the absence of enzyme. These values were subtracted from the corresponding values determined in the presence of enzyme (cf. the curves shown in Fig. 3, below).

The rate of mercaptide formation of S-MDH in the absence and in the presence of 2.6 M or 6 M urea (Fig. 4, below) was determined spectrophotometrically with a Gilford model 2000 multiple sample absorbance recorder. The changes in absorbance were recorded immediately after the addition of known quantities of enzyme to the reaction mixtures containing 6 equivalents of PMB. Other details are given in the legends to the figures.

Spectropolarimetric Measurements

Optical rotatory dispersion studies were carried out with a Cary model 60 spectropolarimeter. A cell of 1-cm path length was used for all measurements; the temperature in the cell compartment was maintained at 27°C. The slit widths of the polarimeter were programmed to give a half-band width of 1.5 µm or less over the entire spectral range studied. The mean residue rotations [m] of S-MDH and M-MDH were calculated with the use of mean residue weights of 114 and 109, respectively, and the refractive index corrections of water and urea solutions as reported in the literature (23). The α₀ and β₀ values were calculated with the Moffitt-Yang equation (24), with a λ₀ value of 212 µm. The λ₀ values were obtained from the single term Drude equation (25).

Results

The effect of preliminary incubation in urea on the activity of S-MDH is shown in Fig. 1. At concentrations of 3 M urea and above, rapid inactivation of the enzyme occurs. Complete loss of enzymic activity is evident in less than 15 min after exposure of the protein to 6 M urea. In 2.6 M urea, after a rapid initial phase of limited inactivation, over 80% of the activity still remains after a period of 4 hours. Assays carried out in the presence of 2.6 M urea yielded similar results. Thus, the results of the preliminary incubation experiment in 2.6 M urea cannot be attributed to a reversal of inactivation due to dilution of the urea in the course of the assay procedure.

Native S-MDH has previously been shown to react with 3 equivalents of PMB with no loss of catalytic activity (18). If this form of active enzyme, designated S-MDH-(PMB)₃, is incubated in 2.6 M urea, however, a gradual loss of enzymatic activity is observed, as shown in Fig. 2. This inactivation is partially prevented by the initial incorporation in the urea solution of cysteine, reduced glutathione, mercaptoethanol, or dithiothreitol (in order of increasing effectiveness) (Fig. 2). In addition, in 2.6 M urea, the 3 "nonreactive" sulfhydryl groups of native S-MDH became accessible to PMB. Thus, as seen in Fig. 3, the full complement of 6 sulfhydryl groups of S-MDH react with this thiol reagent in the presence of 2.6 M urea.

![Fig. 1](http://www.jbc.org) Effect of preliminary incubation in urea on S-MDH activity. S-MDH (3 x 10⁻⁴ M) in 0.1 M potassium phosphate buffer, pH 6.9, was incubated with the indicated concentrations of urea at 25°C. Aliquots of the reaction mixtures were removed at various time intervals, diluted, and immediately assayed for activity ▲, 2.6 M urea; △, 3.0 M urea; ◆, 4.0 M urea; and ●, 6 M urea.
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FIG. 2. Effect of various thiol reagents on the rate of S-MDH.
(PMB) inactivation in 2.6 M urea. S-MDH (5 × 10^{-6} M) in 0.1 M potassium phosphate buffer, pH 6.9, was allowed to react with 3 equivalents of PMB and the reaction was monitored spectrophotometrically at 255 nm to ascertain completion of mercaptide formation (cf. “Experimental Procedure”). After 10 min, an 8 M urea solution containing the specified thiol reagent was added to give a final concentration of 2.6 M urea and 10^{-5} M thiol reagent. Aliquots of each reaction mixture were removed at the indicated time intervals, diluted, and immediately assayed for activity. •, dithiothreitol; O, mercaptoethanol; ●, reduced glutathione; △, cysteine; □, no thiol reagent present; ■, S-MDH (not allowed to react with PMB) in 2.6 M urea, run as a control.

Although 6 equivalents of PMB react with S-MDH in 6 M and also in 2.6 M urea (Fig. 3), the rates of mercaptide formation and attendant loss of enzymatic activity differ significantly depending on the urea concentration, as shown in Fig. 4. In 6 M urea and in the presence of 6 equivalents of PMB the full complement of the S-MDH sulfhydryl groups react within 3 min after addition of the enzyme, with a corresponding complete loss of catalytic activity. The native protein (in the absence of urea) reacts with only 3 equivalents of PMB, and mercaptide formation is complete in 5 min. Under these conditions the enzyme retains its full activity for prolonged periods of time (18). In 2.6 M urea, however, 3 sulfhydryl groups are titrated within 4 min. After this initial, rapid phase of mercaptide formation, an additional 15 min is required for the reaction with the remaining 3 sulfhydryl groups to reach completion. The corresponding residual enzymatic activities after 3.8, 4.7, and 5.6 equivalents of PMB interacted with the S-MDH in 2.6 M urea are 82, 58, and 44%, respectively. At 20 min, with all 6 sulfhydryl groups blocked by PMB, 31% of the initial activity is still retained with complete inactivation evident only after 90 min.

Optical rotatory dispersion studies were carried out to monitor changes in the three-dimensional protein structure accompanying the selective blocking of thiol groups. The optical rotatory dispersion curve for native S-MDH (Fig. 5) is characterized by a distinct shoulder in the 280 to 290 nm spectral region, and a trough near 233 nm ([m]_233 = -5600). Optical rotatory dispersion parameters, calculated by use of standard treatments of the data, are shown in Table II and, considering variations in experimental conditions, are in close agreement with corresponding values of b and λ previously reported for bovine heart S-MDH (1). In 2.6 M urea the shape of the optical rotatory dispersion curve of S-MDH differs slightly from that of the curve obtained with the native enzyme. Pronounced changes in the rotatory properties are obtained however, with S-MDH incubated for 4 hours in a 2.6 M urea solution containing 3 equivalents of PMB or with S-MDH·(PMB)₃ incubated in 2.6 M urea until the same low level of constant residual activity is attained. Under these conditions, the 280 nm shoulder completely disappears and the 233 nm trough is significantly diminished (Fig. 5). In addition, the rotatory parameters as shown in Table II change in a manner suggestive of a gross unfolding of the protein molecule. The rotational changes induced by addition of 6 equivalents of PMB to the enzyme in 2.6 M urea are even more pronounced, although a further degree of unfolding occurs with enzyme in 6 M urea alone.

The time-dependent changes in rotation at 350 nm are a convenient index of the PMB-induced three-dimensional structural modifications of S-MDH in 2.6 M urea. As seen in Fig. 6,
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**FIG. 4.** Rate and stoichiometry of PMB interaction with S-MDH and effect on enzymatic activity. S-MDH was added to give a final concentration of $3 \times 10^{-4}$ M to the various solutions (with or without urea) containing 6 equivalents of PMB in 0.1 M potassium phosphate buffer, pH 6.9. The changes in absorbance at 255 nm were immediately and continuously recorded. Aliquots of the reaction mixtures were removed at the specified time intervals, diluted, and assayed for activity. $\circ$, kinetics of mercaptide formation; $\cdots$, corresponding rates of enzyme inactivation. $\bigcirc$, no urea, full enzymatic activity retained (cf. text and Reference 18); $\triangle$, 2.6 M urea; $\bullet$, 6 M urea.

**TABLE II**

Optical rotatory dispersion parameters of supernatant malate dehydrogenase

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<tr>
<th>Conditions of measurement</th>
<th>$-b_9$</th>
<th>$-a_9$</th>
<th>$\lambda_c$</th>
<th>$[m]_226$</th>
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<tr>
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<td>90</td>
<td>260</td>
<td>3600</td>
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<tr>
<td>S-MDH plus 2.6 M urea, pH 6.9</td>
<td>200</td>
<td>110</td>
<td>250</td>
<td>5400</td>
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<tr>
<td>S-MDH plus 2.6 M urea, pH 6.9, plus 3 equivalents of PMB</td>
<td>105</td>
<td>450</td>
<td>226</td>
<td>4700</td>
</tr>
<tr>
<td>S-MDH plus 2.6 M urea, pH 6.9, plus 6 equivalents of PMB</td>
<td>50</td>
<td>550</td>
<td>220</td>
<td>3900</td>
</tr>
<tr>
<td>S-MDH plus 6 M urea, pH 6.9</td>
<td>10</td>
<td>650</td>
<td>215</td>
<td>3150</td>
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</table>

**FIG. 5.** Optical rotatory dispersion curves of S-MDH under various experimental conditions. The protein concentrations were $1.6 \times 10^{-5}$ M for the studies in the spectral range from 600 to 250 nm, and $3.2 \times 10^{-4}$ M for the measurements in the spectral range from 250 to 225 nm (inset). All reaction mixtures contained, in addition to the components listed below, 0.1 M potassium phosphate buffer, pH 6.9. The enzyme was incubated in the urea and urea-mercurial solutions for 4 hours prior to spectropolariometric analysis; the numbers in parentheses represent the percentage residual enzymatic activity. $\cdots$, S-MDH (native enzyme); $\cdots\cdots$, S-MDH in 2.6 M urea (83%); $\cdots\cdots\cdots$, S-MDH in 2.6 M urea containing 3 equivalents of PMB (22%); $\cdots\cdots\cdots\cdots$, S-MDH in 2.6 M urea containing 6 equivalents of PMB (0%).

**FIG. 6.** Time course of PMB-induced changes in the molar rotation at 350 nm of S-MDH (in urea) with corresponding decreases in enzymatic activity. S-MDH ($1.6 \times 10^{-4}$ M) was incubated with the indicated amounts of PMB in 0.1 M potassium phosphate buffer, pH 6.9, containing 2.6 M urea. At the time indicated by the arrow, dithiothreitol was added to a final concentration of $10^{-3}$ M. Aliquots of the reaction mixtures were removed at various time intervals, diluted, and immediately assayed for activity. $\bullet$, molar rotations at 350 nm and enzymatic activity of S-MDH in 2.6 M urea in the presence of 3 equivalents of PMB; $\blacksquare$, molar rotations at 350 nm and enzymatic activity of S-MDH in 2.6 M urea in the presence of 6 equivalents of PMB.

the rotational changes in the presence of 3 equivalents of PMB are essentially complete in 4 hours. Losses of enzymatic activity over the same period of time closely parallel these changes in rotation. After 6 hours, with a low and constant level of residual catalytic activity still remaining, the addition of dithiothreitol results in a partial reactivation of the enzyme with a corresponding restoration of the rotati9nal properties. More rapid changes in rotation and attendant parallel losses in enzymatic activity are observed after addition of 6 equivalents of PMB to S-MDH in 2.6 M urea. In addition, the enzyme is completely and irreversibly inactivated within 14 hours.

The results of a comparable study of the optical rotatory dispersion properties of bovine heart M-MDH are summarized in Fig. 7 and Table III. The calculated optical rotatory parameters presented here are in fair agreement with those reported for
In the present study an attempt has been made to delineate further the differences in reactivity of the sulfhydryl groups of S-MDH in relation to its structure and catalytic function. In the native state, only 3 of the 6 sulfhydryl groups of S-MDH react rapidly with PMB with no loss of enzymatic activity. \(^*\) This is in contrast to a number of other enzymes (8, 0, 20, 30) that are inactivated by the selective blocking of specific reactive sulfhydryl groups. Experimental conditions have now been defined in which the interaction of S-MDH with 3 equivalents of PMB results in loss of enzymatic activity and in profound structural changes in the protein molecule.

It has been shown that prolonged incubations of S-MDH in 2.6 M ureas do not alter significantly the catalytic properties of the enzyme. Although 3 equivalents of PMB or 2.6 M urea acting independently on S-MDH do not significantly affect the enzymatic activity, S-MDH·(PMB)\(_3\) in 2.6 M urea is gradually inactivated and profound changes in optical rotatory dispersion properties are discernible. In addition, all 6 sulfhydryl groups of S-MDH ultimately become accessible for reaction with PMB in 2.6 M urea. The optical rotatory dispersion studies are consistent with the view that 2.6 M urea induces a slight change in the three-dimensional structure of the protein and that the molecule thus becomes progressively susceptible to additional conformational changes resulting from reaction with PMB.

Optical rotatory dispersion techniques have been used extensively for the determination of protein structure (31-33), and decreases in the magnitude of the trough near 233 \(\text{nm}\) and in \(\Delta\beta\) and \(\lambda_\alpha\) values have generally been interpreted as being indications of protein unfolding. These rotatory criteria have been applied in the present study to detect and evaluate conformational changes in the two bovine heart malate dehydrogenases. In the presence of urea or PMB or both, the results are suggestive of significant alterations in the ordered or helical structures in the protein molecule. In addition, the characteristic shoulder in the 280 to 290 \(\text{nm}\) spectral region in the optical rotatory dispersion curve of S-MDH may be used as an index of the conformational features of this protein. A shoulder in this spectral region has now been reported for a large number of proteins (34–38), and has generally been considered to represent as intrinsic Cotton effect ascribed to specific spatial alignments of aromatic amino acid residues. In this connection, it is noteworthy that M-MDH, which contains little (18) or no (39, 40) tryptophan (and slightly less tyrosine than S-MDH (18)), exhibits a plain dispersion curve in this spectral region. The calculation of the Moffitt-Yang constants may be influenced by the magnitude of the 280 \(\text{nm}\) shoulder because the effects of such an inflection point are not considered in the derivation of their equation (24, 34). Therefore, these values as reported in the present study are not to be construed as absolute indications of the degree of helicity, but rather as reflecting the over-all conformational changes observed.

Several conformationally discrete forms of S-MDH have been distinguished on the basis of their respective optical rotatory dispersion properties. The native enzyme (\([m^\circ]_{233} -5000\)) is unfolded slightly in 2.6 M urea (\([m^\circ]_{233} -5400\)). The subsequent addition of 3 or 6 equivalents of PMB results in a gradual but ultimately significant degree of unfolding (\([m^\circ]_{233} -4700\) and \([m^\circ]_{233} -4000\)).

\(^*\) The reaction of N-ethylmaleimide with the thiol groups of S-MDH follows a different pattern. N-Ethylmaleimide does not react with the native enzyme. In 2.5 M urea, only 3 equivalents of N-ethylmaleimide react with no significant losses in enzymatic activity (unpublished results). In 6 M urea, as previously reported (18), approximately 6 equivalents of N-ethylmaleimide react with S-MDH and a catalytically inactive form of the enzyme is obtained.

**Discussion**

In the present study an attempt has been made to delineate further the differences in reactivity of the sulfhydryl groups of pig and horse heart M-MDH by Thorne and Kaplan (26), but differ considerably from the divergent values previously reported for various preparations of pig heart M-MDH (27, 28). The shoulder in the 280 to 290 \(\text{nm}\) spectral region that characterizes the optical rotatory dispersion curve of S-MDH (Fig. 5) is conspicuously absent from the curve obtained with native M-MDH (Fig. 7). The other rotatory properties however, are similar for both enzymes. Although the sulfhydryl groups of native M-MDH react very sluggishly with PMB, the full complement of 12 half-cystine residues per molecule of enzyme react ultimately (18). In addition, rapid losses of catalytic activity occur after the enzyme has reacted with only 3 equivalents of PMB. It is therefore not surprising that titration of native M-MDH with 10 equivalents of PMB (in the absence of urea) results in large changes in the optical rotatory dispersion curve and calculated parameters of this enzyme (Fig. 7; Table III).

**Table III**

<table>
<thead>
<tr>
<th>Conditions of measurement</th>
<th>(\lambda_\beta)</th>
<th>(\phi)</th>
<th>(\lambda_\alpha)</th>
<th>([m^\circ]_{233})</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-MDH, pH 7.4</td>
<td>230</td>
<td>145</td>
<td>260</td>
<td>5930</td>
</tr>
<tr>
<td>M-MDH, pH 7.4, plus 10 equivalents of PMB</td>
<td>140</td>
<td>345</td>
<td>238</td>
<td>4900</td>
</tr>
<tr>
<td>M-MDH, pH 7.4, plus 6 M urea</td>
<td>20</td>
<td>800</td>
<td>155</td>
<td>3700</td>
</tr>
</tbody>
</table>

The optical rotatory dispersion parameters of mitochondria malate dehydrogenase

**Fig. 7.** Optical rotatory dispersion curves for M-MDH and for M-MDH in the presence of 10 equivalents of PMB. Protein solutions, \(1.4 \times 10^{-5} \text{M}\), in 0.1 M potassium phosphate buffer, pH 7.4, were used for the studies in the spectral range, 600 to 250 \(\text{nm}\). For measurements below 250 \(\text{nm}\) (inset) the protein was further diluted with buffer to a final concentration of \(2.8 \times 10^{-5} \text{M}\). The M-MDH was incubated with 10 equivalents of PMB for 10 hours at 0°C prior to spectropolarimetric analysis; approximately 10% of the initial activity was retained. ———, M-MDH (native protein); ·····, M-MDH with 10 equivalents of PMB.
In 6 M urea alone, S-MDH exists in a highly disordered state ($[^{1}H]_{M}233 -3150$). The catalytically active, multiple forms of chicken heart mitochondrial malate dehydrogenase have recently been reported to differ from each other only in conformation (41). The electrophoretically distinct "conformers" exhibit a wide range of $[^{1}H]_{M}233$ values. Thus, the form migrating as "Band E" ($[^{1}H]_{M}233 -2144$) with a highly unfolded structure is enzymatically as active as the considerably more ordered form in "Band A" ($[^{1}H]_{M}233 -4890$). On the other hand, it is evident from the results obtained in the present study that extensive inactivation occurs with S-MDH-(PMB)$_3$ in 2.6 M urea with a disruption of less than 25% of the native ordered structure (assuming an $[^{1}H]_{M}233$ value of $-1800$ for the completely random form (42)).

Of the 6 thiol groups in S-MDH, at least 3 are catalytically nonessential, and these are characterized by their accessibility in the native protein for reaction with PMB. Mercaptide formation with these 3 reactive sulfhydryl groups, however, labilizes the enzyme (possibly via a steric mechanism) so that formation with these 3 reactive sulfhydryl groups, however, occurs with the 3 non-reactive thiol groups of S-MDH in 2.6 M urea is always more rapid than the rate of enzymatic inactivation (Fig. 4). In addition, the loss of catalytic activity closely parallels the rate of change of the optical rotatory dispersion properties of the enzyme (Fig. 6). It is therefore apparent that the loss in activity is more closely related to the secondary structural changes induced in the enzyme than to the primary interaction of these sulfhydryl groups with PMB. The involvement of thiol groups in the maintenance of the proper conformation of the active sites of a number of enzymes has been suggested. These include citrate-condensing enzyme (13), pig heart M-MDH (43), frumarase (15), myokinase (11), creatine kinase (44), pyridoxamine pyruvate transaminase (14), DPNH dehydrogenase (45), phosphoglucomutase (46), and phosphoglucoisomerase (47), although direct experimental evidence for conformational changes accompanying blocking of sulfhydryl groups was not presented. From the changes in the optical rotatory dispersion properties reported in the present study it is apparent that significant conformational alterations are induced by the interaction of PMB with the thiol group of the bovine heart malate dehydrogenases.

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REFERENCES

Beef Heart Malic Dehydrogenases: VII. REACTIVITY OF SULFHYDRYL GROUPS AND CONFORMATION OF THE SUPERNATANT ENZYME
Arabinda Guha, Sasha Englard and Irving Listowsky

J. Biol. Chem. 1968, 243:609-615.

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