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Subunits of Rat Liver Mitochondrial Malate Dehydrogenase*

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ABSTRACT: Considerable evidence has suggested that mammalian mitochondrial malate dehydrogenase is composed of subunits. In the present investigation the molecular weight of highly purified rat liver malate dehydrogenase has been studied in several systems by sedimentation equilibrium and by sedimentation diffusion. The intact molecule has a molecular weight of 66,300. The molecule is dissociated at pH 2, or in 5 or 7 M guanidine hydrochloride or 8 M urea to half-molecules. The half-molecules present at pH 2 or in 8 M urea are inactive.

The inactivated enzyme can be reactivated by neutralization in the one case or by removal of the denaturing reagent in the other. The data presented indicate that the extent of reactivation which occurs on neutralization of enzyme solutions exposed to a pH 2 environment is dependent upon the length of time at pH 2. In a pair of sedimentation equilibrium experiments the molecular weight of beef heart malate dehydrogenase was shown to be 64,000 in 0.1 M sodium citrate–0.002 M 2-mercaptoethanol (pH 6.25). In 5 M guanidine hydrochloride a value of 35,700 was obtained.

Purified preparations of rat liver mitochondrial malate dehydrogenase can be separated into five bands of catalytic activity when subjected to starch gel electrophoresis. Of these five isozymes, three are responsible for about 90% of the total catalytic activity. Such malate dehydrogenase isozymes have previously been reported for a number of species (Grimm and Doherty, 1961; Thorne *et al.*, 1963).

A great deal of evidence has accumulated which indicates that malate dehydrogenase is composed of subunits. However, some confusion has arisen over the number of subunits in the molecule.

Munkres and Richards (1965) have reported that the malate dehydrogenase of *Neurospora crassa* is composed of four subunits. Further, Munkres (1965) has shown that the enzyme is composed of two types of polypeptide chains (designated α and β) and has the over-all structure $\alpha_3\beta$. Recently Siegel (1967) has reported that beef heart malate dehydrogenase is composed of four subunits, the molecule being dissociated in 4 M urea.

Dévényi *et al.* (1966) have proposed that the pig heart enzyme is composed of two similar or identical subunits on the basis of tryptic fingerprint studies. Thorne *et al.* (1963) have studied the binding of NADH to the pig heart enzyme, and have determined that there are two binding sites per molecule.

The present paper deals with dissociation studies of rat liver malate dehydrogenase. These studies, conducted in 4 and 8 M urea, in 5 and 7 M guanidine hydrochloride, and in a series of experiments at pH 2.0 indicate that the rat liver malate dehydrogenase molecule is composed of two subunits of equal molecular weight.

Experimental Procedures

Materials

Rat liver malate dehydrogenase was purified by the

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method of Sophianopoulos and Vestling (1962) from frozen rat livers (supplied by Pentex). The enzyme prepared by this method behaves as a single component during ultracentrifugation, diffusion, and free-boundary electrophoresis. The concentration of the protein was determined from the absorbance at 280 mμ; $E^{1\%}_{1cm}$ 5.08. L-Malic acid and NAD for enzymatic assays were purchased from the Sigma Chemical Co.

Urea was purchased from the Sigma Chemical Co. and guanidine hydrochloride from Eastman Organic Chemicals. Both dissociating reagents were used without further purification.

Methods

Assay Procedure. Enzymatic activity was measured spectrophotometrically from the malate side of the equilibrium using a Cary Model 15 spectrophotometer. The assay procedure was that described by Sophianopoulos and Vestling (1962).

Molecular Weight Determination. Sedimentation equilibrium experiments were performed using the short column, high speed technique of Yphantis (1964).

All experiments were performed at 20° using double-sector cells with saphire windows. Samples for study were diluted to a concentration of 0.01–0.02% with the appropriate solution, and dialyzed *vs.* the solution for at least 12 hr.

Appropriate speeds for each experiment were chosen using the speed chart provided by Van Holde (1967) or calculated using the equation, $\sigma = \omega^2 s/D$, where ω = the angular velocity of the rotor, and $\sigma = \omega^2 M(1 - \bar{v}\rho)/RT$. In accordance with Yphantis' suggestion, σ was set at approximately 5 cm^{-2}.

Estimations of the equilibrium times were made using the equation $t_{equil} = 2.3(ba)/\omega^2 sr$, where t_{equil} is the length of time required for the establishment of equilibrium, $b - a$ is the solution column height, and r is the radius. As a check to determine whether equilibrium had been established, the fringe displacements at given x coordinates were measured on photographs taken at least 2 hr before the final photographs and the values obtained compared with the final values.

The average rotor velocity during the experiment was determined directly through the use of the odometer, and an electronic timer. The rotor velocities obtained experimentally were in excellent agreement with the indicated velocities.

Sophianopoulos (1962) has determined the partial specific volume, $\bar{v}$, of native rat liver malate dehydrogenase to be 0.734 cc/g, and this value has been used in the calculation of molecular weights. The value of $\bar{v}$ to be used in guanidine hydrochloride or urea solutions, however, presents a problem. Kielly and Harrington (1960) have reported that the $\bar{v}$ of myosin decreases by 0.01 cc/g in 5 M guanidine hydrochloride, and Marler and Tanford (1964) have reported a similar change in $\bar{v}$ for γ-globulin in this solvent. On the other hand, Reithel and Sakura (1963) have reported little change in $\bar{v}$ for a number of proteins in guanidine. We have considered two possibilities for $\bar{v}$ in computing protein molecular weights in denaturing solvents. The molecular weights presented in this paper are based on: (1) the assumption that there is no change in $\bar{v}$ in a denaturing solvent ($\bar{v}$ = 0.734 cc/g) and (2) $\bar{v}$ in a denaturing solvent decreases by 0.01 cc/g ($\bar{v}$ = 0.724 cc/g). Neither choice of $\bar{v}$ presents difficulty in making an assignment of the number of subunits per molecule for rat liver malate dehydrogenase.

Sedimentation coefficients were obtained in the usual manner. Approximate diffusion coefficients were estimated from the schlieren pattern, photographed at high speed using the maximum area ordinate method described by Schachman (1957). In all ultracentrifugal experiments the densities and viscosities of solvents were determined directly.

Results

Molecular Weight of Native Rat Liver Malate Dehydrogenase. The rapid equilibrium technique of Yphantis (1964) was applied to a solution containing 0.01% rat liver malate dehydrogenase of maximal specific activity in 0.1 M sodium citrate–0.002 M 2-mercaptoethanol, pH 6.25. The rotor speed in this experiment was 29,500 rpm, and the final photograph was taken 23 hr after reaching speed. The graphical interpretation of the data in terms of a plot of the log of the Raleigh fringe displacement, Y, *vs.* the square of the distance in centimeters from the center of rotation, r, is shown in Figure 1. The material is homogeneous under these experimental conditions and has a molecular weight of 66,300. This value is in good agreement with that reported by Siegel (1967) for beef heart malate dehydrogenase (65,000) but differs somewhat from the value obtained by Burck (1962) for rat liver malate dehydrogenase as calculated from sedimentation diffusion data (59,800) and that reported by Thorne *et al.* (1963) for pig heart malate dehydrogenase (70,000). The value of 66,300 will be used as the molecular weight of the native rat liver malate dehydrogenase molecule throughout this paper.

Dissociation of the Rat Liver Malate Dehydrogenase Molecule at pH 2. Rat liver mitochondrial malate dehydrogenase in 0.1 M 2-mercaptoethanol was inactivated by titration with 0.028 M HCl–0.1 M 2-mercaptoethanol according to the method of Chilson *et al.* (1965, 1966). The inactivated enzyme could be reactivated by dilution with 0.5 M sodium citrate–0.1 M 2-mercaptoethanol (pH 7.0). Inactivation was carried out at 0° while reactivation was carried out at 20°.

During reactivation studies involving the method of Chilson *et al.*, it was observed that the extent of reactivation appeared to depend upon the length of time the enzyme was exposed to the pH 2.0 environment. In order to assess the effect of time of exposure at pH 2.0, the following experiment was performed. Samples of malate dehydrogenase in 0.1 M 2-mercaptoethanol were rapidly titrated to pH 2.0 with predetermined volumes of 0.028 M HCl–0.1 M 2-mercaptoethanol in an ice bath. At the end of predetermined intervals, the samples were diluted with appropriate volumes of 0.5 M sodium citrate–0.1 M 2-mercaptoethanol (pH 7.0) which had been preequilibrated at 20°, and the vessels were transferred to a 20° water bath. The return of enzymatic activity was monitored by withdrawing aliquots and assaying

for enzymatic activity in the conventional manner.[1] The results of this experiment are shown in Figure 2. It can be seen that both the rate and extent of reactivation were influenced by the length of time the enzyme had remained at pH 2.0.

The inactivated enzyme at pH 2 was subjected to sedimentation analysis in order to determine whether the acidification process had caused dissociation. The material sedimented as a single peak with a sedimentation coefficient of 0.955 S. The same enzyme sample was permitted to stand for 5 days at 4° and was again subjected to sedimentation analysis. The material again sedimented as a single peak, having an $s_{20,w}$ of 0.986 S. From these data it was concluded that no gross changes in the molecular size of the subunit were occurring. Dilution of this enzyme solution with 0.5 M sodium citrate (pH 7.0) led to a recovery of 8% of the initial activity.

Sedimentation studies of the malate dehydrogenase molecule under acid dissociating conditions were complicated by the fact that the addition of neutral electrolytes caused aggregation and precipitation of the acidified protein. As a consequence, initial sedimentation studies were conducted in the absence of added electrolyte; thus, the sedimentation velocities reported must be treated with caution as they represent hydrodynamic properties obtained under conditions where intermolecular interactions are not minimized. The protein molecule is no doubt highly charged at pH 2.0, and the ionic strength of the supporting medium is low. Such a situation has the effect of giving a low value for the sedimentation coefficient, and a high value for the diffusion coefficient. Consequently, a low value would result if one calculated a molecular weight from these parameters obtained under such conditions.

The rapid sedimentation equilibrium technique of Yphantis (1964) is applied to protein solutions of much lower concentrations than those used in sedimentation–diffusion experiments. At low protein concentration, intermolecular interactions are minimized, and it was thought that the sedimentation equilibrium technique might yield a reasonable estimate of the molecular weight of the malate dehydrogenase subunit.

A sedimentation equilibrium experiment was performed on a 0.01% solution of malate dehydrogenase in 0.01 M HCl–0.1 M 2-mercaptoethanol–2% sucrose (pH 2.0). In this experiment sucrose was added to increase the density and viscosity of the solvent in order to stabilize the concentration gradient with respect to convection. The equilibrium experiment was performed at 42,040 rpm for 24 hr at 20°. There was evidence of some convective erosion of the leading edge of the gradient; however, a sufficient portion of the gradient remained intact for the estimate of the molecular weight. The value obtained in this experiment was 29,700. This molecular weight would indicate that the molecule is dissociated into two subunits at pH 2. Five similar experiments were performed.

Molecular Weight of the Subunit in Guanidine Hydrochloride. In order to obtain a subunit molecular weight under dissociating conditions where charge effects are minimized, it was decided to determine the minimal molecular weight for the subunits in guanidine hydrochloride.

Hemoglobin (Kawahara *et al.*, 1965) and lactate dehydrogenase (Appella and Markert, 1961) have been shown to be completely dissociated by 5 M guanidine hydrochloride. This concentration (5 M) of guanidine hydrochloride was chosen to effect dissociation of malate dehydrogenase. A solution of malate dehydrogenase of maximal specific activity was diluted to a concentration of 0.01% with a solution containing 5 M guanidine hydrochloride–0.02 M sodium citrate–0.1 M 2-mercaptoethanol (pH 6.25) and dialyzed *vs.* the same buffer for 24 hr. The equilibrium experiment was run at 42,040 rpm for 26.5 hr at 20°. The data obtained from the ex-

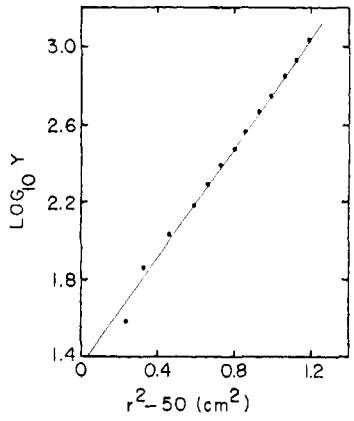

FIGURE 1: Plot of log displacement, Y, *vs.* square of distance from center of rotation, r. Sedimentation equilibrium data for a solution of rat liver malate dehydrogenase (0.01% in 0.1 M sodium citrate–0.002 *M* 2-mercaptoethanol (pH 6.25)). The experiment was carried out for 20 hr at 20° and 29,500 rpm. The line is drawn from a least-squares analysis which includes all fringe displacements greater than 25 μ.

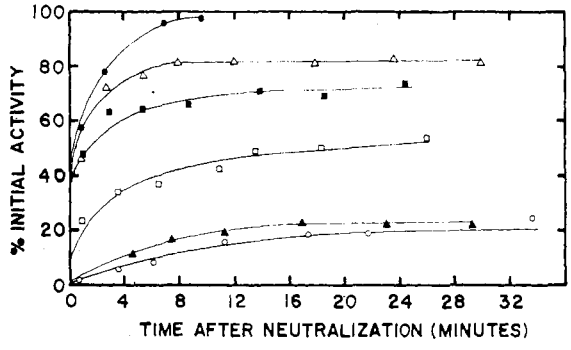

FIGURE 2: Rate of recovery of enzymatic activity following inactivation at pH 2. Inactivation was accomplished by titration of malate dehydrogenase solutions (3 mg/ml of malate dehydrogenase–0.1 M 2-mercaptoethanol) to pH 2 with equal volumes of 0.028 M HCl–0.1 M 2-mercaptoethanol at 0°. The acidified enzyme solutions were permitted to remain at pH 2, 0° for varying time intervals (●, 2.15 min; △, 4.92 min; ■, 9.75 min; □, 22.0 min; ▲, 71.12 min; ○, 664 min). Neutralization was accomplished by a 100-fold dilution of the acidified enzyme solutions with 0.5 M sodium citrate–0.1 M 2-mercaptoethanol (pH 7.0) at 20°.

[1] This study was made possible by the fact that reactivation of the enzyme did not occur under the conditions in the assay cuvet.

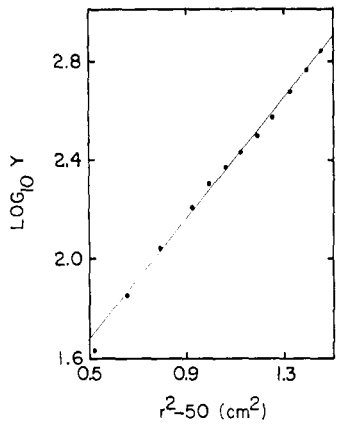

FIGURE 3: Plot of log fringe displacement, Y, *vs.* the square of the distance from the center of rotation, r. Sedimentation equilibrium data for a solution of rat liver malate dehydrogenase (0.02% in 7 M guanidine hydrochloride–0.1 M 2-mercaptoethanol–0.02 M sodium citrate (pH 6.25)). The experiment was carried out for 28 hr at 20° and 52,346 rpm. The line is drawn from a least-squares analysis which includes all fringe displacements greater than 70 μ.

periment (assuming the partial specific volume to be 0.734 cc/g) led to a value for the molecular weight of 32,200.

A second equilibrium experiment in 5 M guanidine hydrochloride on a sample prepared in the same fashion was performed at 50,740 rpm for 23 hr. The data obtained from this run gave a molecular weight of 33,500.

Recently, Paetkau *et al.* (1968) have reported that phosphofructokinase is not completely dissociated in 5.5 M guanidine hydrochloride. In view of this report a sedimentation equilibrium experiment on rat liver malate dehydrogenase was conducted in 7 M guanidine hydrochloride–0.1 M 2-mercaptoethanol in order to test whether further dissociation of the rat liver malate dehydrogenase molecule was possible. This experiment was conducted on a 0.02% malate dehydrogenase solution for 28 hr at 52,346 rpm. The graphical data are shown in Figure 3, and a molecular weight of 31,100 was obtained.

Dissociation of the Rat Liver Malate Dehydrogenase Molecule in Urea. Preliminary studies have been conducted on rat liver malate dehydrogenase in urea. The rat liver enzyme is inactivated to the extent of 90% in 4 M urea. However, approximate molecular weights obtained by sedimentation–diffusion indicate that the rat liver enzyme does not dissociate in 4 M urea. Under these conditions, a molecular weight of 61,000 was obtained. A similar analysis performed on pig heart malate dehydrogenase, however, indicated that this molecule does dissociate in 4 M urea. An approximate molecular weight of 29,000 for the pig heart enzyme was obtained from sedimentation diffusion data.

Rat liver malate dehydrogenase is dissociated in 8 M urea. Combination of sedimentation–diffusion data obtained in the ultracentrifuge in the Svedberg equation gave a molecular weight of 32,900 for the malate dehydrogenase molecule in 8 M urea. Rat liver malate dehydrogenase was exposed to 8 M urea in 0.1 M 2-mercaptoethanol–0.02 M sodium citrate (pH 6.25) for 30 min at 5° and then dialyzed *vs.* 0.5 M sodium citrate–0.1 M 2-mercaptoethanol (pH 7.0) for 12 hr. The resulting sample contained 54% of its initial activity.

TABLE I: Molecular Weights of Rat Liver Malate Dehydrogenase and Malate Dehydrogenase Subunits.

Conditions (M), rpm	Mol Wt ($\bar{v}_1$)[a]	Mol Wt ($\bar{v}_2$)[b]
Sodium citrate (0.1), 29,500	66,300 ± 1,000	
Hydrochloric acid (0.01), 42,040	29,700 ± 400	
Guanidine hydrochloride (5), 42,040	32,200 ± 700	30,300 ± 600
Guanidine hydrochloride(5), 50,740	33,500 ± 500	31,400 ± 400
Guanidine hydrochloride (7), 52,346	31,100 ± 500	28,800 ± 400
Urea (8) (sedimentation diffusion)	32,900	

[a] $\bar{v}_1 = 0.734$ (native). [b] $\bar{v}_2 = 0.724$.

Table I gives a compilation of the molecular weights obtained for the malate dehydrogenase molecule under the dissociating conditions described in this paper.

Molecular Weight of Beef Heart Malate Dehydrogenase and Its Subunits. Molecular weight determinations were performed on a sample of beef heart malate dehydrogenase kindly supplied by Dr. L. Siegel. The high-speed equilibrium technique gave a molecular weight of 64,000 for the native molecule under the same buffer conditions used for rat liver malate dehydrogenase. The dissociation of the molecule in 5 M guanidine hydrochloride resulted in a species with a molecular weight of 35,700. These data indicate that the beef heart malate dehydrogenase molecule is also composed of two subunits. This result contrasts with the suggestion of Siegel (1967) that beef heart malate dehydrogenase can be dissociated into four subunits in 0.2 M sodium citrate, pH 2.6, 5°, as determined by sedimentation diffusion experiments. It should be recognized that the conditions employed by Siegel are different from those used with beef heart malate dehydrogenase in the present report. However, the data in Table I in the present report demonstrate that in the case of rat liver malate dehydrogenase the subunit molecular weight does not vary with such conditions

Discussion

The data presented in this paper indicate that the rat liver malate dehydrogenase molecule is composed of two subunits of equal molecular weight. Even under dissociating conditions as strenuous as 7 M guanidine hydrochloride no component with a molecular weight less than half of that of the whole molecule was observed.

The lack of an appropriate $\bar{v}$ in guanidine hydrochloride does not lead to any uncertainty in the determination of the number of subunits in the malate dehydrogenase molecule. Subunit molecular weights calculated on the basis of an assumed maximum $\bar{v}$ change in guan-

idine hydrochloride lead in the most extreme case (7 M guanidine hydrochloride) to a molecular weight of 28,800. This value would give 2.3 subunits per malate dehydrogenase molecule. The values for the subunit molecular weight computed on the basis of an unchanged $\bar{v}$ give the best correlation with an integral value for the subunit molecular weight (33,150), and one is tempted to suggest that the $\bar{v}$ of malate dehydrogenase is changed very little in guanidine hydrochloride. The molecular weights determined in guanidine hydrochloride and computed using the native $\bar{v}$ give an average molecular weight of 32,300 or 2.05 subunits per molecule.

The value for the subunit molecular weight determined for the rat liver malate dehydrogenase molecule dissociated at pH 2 (29,700) indicates that complete dissociation to half molecules takes place at pH 2. The fact that the subunit molecular weight determined at pH 2 is slightly low (2.23 subunits/molecule) probably reflects the nonideality of the system in which the equilibrium gradient was established.

The studies of the dissociation process occurring when the rat liver malate dehydrogenase molecule is exposed to a pH 2 environment have not been designed to provide a precise estimate of the time course of the dissociation process. Enzymatic activity has not been detected in aliquots of acidified enzyme solutions which have been assayed within 1 min after acidification. Thus, it is known that inactivation is very rapid. However, one cannot equate inactivation with dissociation. While the time required to dissociate the enzyme is not accurately known, it is certain from sedimentation velocity experiments that the enzyme is completely dissociated within 15 min after acidification. The work of Anderson and Weber (1966) on the acid dissociation of lactate dehydrogenase indicates that this enzyme is completely dissociated within 20 sec after acidification.

The observation that the yield of enzymatic activity on neutralization after acidification is dependent upon the length of time the enzyme is exposed to a pH 2 environment is similar to that reported by Anderson and Weber for the acid dissociation of lactate dehydrogenase. However, this observation is contrary to the findings of Chilson *et al.* (1965) who reported that neither the rate nor degree of reactivation of pig heart malate dehydrogenase was influenced by the length of time the enzyme was exposed to acid pH. This can most likely be explained by the fact that beyond a certain time interval (60 min) there is little change in the rate or degree of reactivation.

The data in the present paper indicate that a slow reaction involving perhaps an irreversible unfolding of malate dehydrogenase subunits is responsible for the losses incurred during acid inactivation. From previous studies in this laboratory (Mann, 1967) it has been observed that malate dehydrogenase can be completely inactivated without dissociation by titration to pH 4. The loss of activity at pH 4 is a relatively slow first-order process. The enzyme can be reactivated by titration to neutral pH, and again the return of activity is first order.

On the basis of the studies of the inactivation of malate dehydrogenase at pH 4 and at pH 2 and a knowledge of the subunit structure of malate dehydrogenase, one can speculate on a possible mechanism of the inactivation and reactivation of the enzyme following acidification and neutralization. The loss of activity at pH 4 is probably due to conformational changes affecting the enzyme active site caused by the increased net positive charge on the molecule. When the enzyme is titrated to pH 4 and then to pH 2, one can write

$$E \underset{pH7}{\overset{pH4}{\rightleftharpoons}} E^* \underset{pH4}{\overset{pH2}{\rightleftharpoons}} 2\,[E^*/2] \longrightarrow 2\,[E_i/2]$$

where E* is inactive whole enzyme and E_i is irreversibly inactivated enzyme. At pH 2 the inactivated whole molecule dissociates into half molecules. The dissociation step is followed by a rather slow consecutive reaction to a form of half molecule which has lost the capability of being reactivated. On titration to pH 7 enzyme in the form of 2 [E*/2] returns to its native state, E, while enzyme in the form of 2 [E_i/2] fails to be reactivated.

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