Interaction of Mitochondrial Malate Dehydrogenase Monomer with Phospholipid Vesicles

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The association between bovine and porcine mitochondrial malate dehydrogenase (EC 1.1.1.37) and phospholipid vesicles was investigated. At concentrations at which malate dehydrogenase exists as a dimer, entrapment within the aqueous compartment but not binding of the ^{14}C -labelled enzyme was observed. The dissociated enzyme was labile to moderate heat and to *p*-chloromercuribenzoate, but in both cases inactivation was decreased by incubation with suspensions of charged phospholipid vesicles. This suggested an interaction between enzyme subunits and phospholipid, and this was confirmed by direct binding measurements and by studies that followed changes in the fluorescein-labelled enzyme. The circular-dichroism spectra of the enzyme indicated a high α -helix content, and suggested that a small conformational change occurred when the enzyme dissociated. Fluorescence data also suggested less-rigid molecules after dissociation. A possible mechanism, based on the flexibility of enzyme monomer and its interaction with phospholipids, by which mitochondrial matrix enzymes are specifically localized in cells, is discussed.

Investigations of the interactions between purified lipids and proteins have demonstrated that maintenance of the tertiary and quaternary structures of many native proteins is dependent on the binding of specific lipid components (Rothfield & Romeo, 1971; Razin, 1972; Papahadjopoulos & Kimelberg, 1973). Studies on both integral membrane proteins and water-soluble proteins have shown that lipids and proteins can interact ionically and hydrophobically (Bigelow, 1967; Capaldi & Vanderkooi, 1972; Papahadjopoulos & Kimelberg, 1973). Membrane-bound proteins usually have both types of interaction (Zwaal & van Deenen, 1970; Lenaz *et al.*, 1970; Singer & Nicholson, 1972), and it has been shown in many cases that integral membrane enzymes require specific lipids to be active *in vitro* (Awasthi *et al.*, 1971; Pitotti *et al.*, 1972; Kimelberg & Papahadjopoulos, 1972; Roelofsen & van Deenen, 1973). The initial interaction with soluble proteins is usually ionic, and the ionic interaction may be followed by hydrophobic binding (Kimelberg & Papahadjopoulos, 1971*a,b*). It is possible that when certain soluble proteins interact with membrane phospholipids an initial ionic interaction may induce a conformational change in the protein that causes more hydrophobic groups to become exposed, and these groups may then penetrate the membrane

interior (Gulik-Krzywicki *et al.*, 1969; Hammes & Schullery, 1970; Dickerson *et al.*, 1971; Kimelberg & Papahadjopoulos, 1971*b*). From studies on these interactions and others, including those on plasma lipoproteins (Assmann & Brewer, 1974; Andrews *et al.*, 1976), apolipoprotein III (Pownall *et al.*, 1974; Novosad *et al.*, 1976), glucagon (Epand *et al.*, 1977*a,b*) and others (Marchesi *et al.*, 1972; Segrest *et al.*, 1976), amphipathic helix models have been developed to explain the penetration of the membrane by certain proteins that can refold to expose a hydrophilic or hydrophobic surface in response to a change in their environment (Spatz & Strittmatter, 1971).

In the present paper we describe studies on the interactions between phospholipid vesicles and mitochondrial malate dehydrogenase (EC 1.1.1.37). Although this enzyme can interact with fatty acid monolayers under some conditions (Peters & Fromherz, 1975), we present data to show that the native enzyme does not interact with phospholipid vesicles. In contrast, malate dehydrogenase subunits have considerably increased stability when incubated with charged phospholipid vesicles, suggesting that the subunits interact with phospholipids. An association was also demonstrated by several other experimental approaches. It is possible that the interactions described may be associated with the exposure of groups on malate dehydrogenase subunits that are

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normally not exposed in the native enzyme. If the exposure of these groups on the enzyme subunits is required to initiate interaction between the enzyme and phospholipid, then this interaction may relate to the question of the specific localization of mitochondrial matrix enzymes and their transport across mitochondrial membranes.

Experimental

Materials

Phosphatidylcholine and phosphatidylserine were purified from egg yolk and bovine brain respectively as described previously (Papahadjopoulos *et al.*, 1973) and stored under nitrogen in sealed ampoules at -20°C until used. Stearylamine was purchased from Fluka A.G. (Buchs, Switzerland); dipalmitoyl phosphatidylcholine and cholesterol were from Sigma Chemical Co., St. Louis, MO, U.S.A. All phospholipids were over 99% pure as estimated by t.l.c. A crude preparation of mitochondrial phospholipid was prepared by using mitochondria from male Wistar rats (200–250 g) as described by Marinetti *et al.* (1958), and the composition determined by t.l.c. Mitochondrial malate dehydrogenase (EC 1.1.1.37) was from Sigma (bovine; lot 72C-878404) or Miles-Seravac, Elkhart, IN, U.S.A. (porcine; batch 95AB), and was the highest specific enzyme activity available. [^{14}C]Formaldehyde (12 mCi/mmol), Phase Combining System scintillation 'cocktail' and D-[U- ^{14}C]glucose (317 mCi/mmol) were purchased from Amersham/Searle Corp., Arlington Heights, IL, U.S.A.; di[^3H]palmitoyl phosphatidylcholine (4 Ci/mmol) was prepared as described earlier (Papahadjopoulos *et al.*, 1974); oxaloacetic acid and NADH were from Sigma. All other reagents were of the highest purity available.

Methods

Enzyme purification. The commercially purified mitochondrial malate dehydrogenase was subjected to a further purification step. The freshly dialysed enzyme, in 100 mM-sodium/potassium phosphate buffer at 4°C , was mixed with five times its own weight of phosphatidylserine/phosphatidylcholine (1:1, w/w) and the phospholipid was then separated from free enzyme by centrifugation at $40000g_{\text{av}}$ for 40 min. Although enzyme activity in the supernatant remained constant during this treatment, a significant but variable amount of protein became irreversibly bound to the phospholipid fraction. The purification step was repeated immediately before each experiment to remove enzymically inactive protein that reaccumulated during storage. This protein was probably denatured enzyme. Reaccumulation could be largely prevented if 5 mM-NADH and 5 mM-oxaloacetate

were present in the storage medium. Binding of coenzyme and substrate probably stabilized the enzyme (Friedman *et al.*, 1975).

Labelling of malate dehydrogenase. Mitochondrial malate dehydrogenase was labelled by reductive methylation to a specific radioactivity of between 1×10^6 and 2×10^6 c.p.m./mg of protein, by using [^{14}C]formaldehyde exactly as described by Rice & Means (1971). The labelling caused no loss of enzyme activity. Labelling with fluorescein was essentially as described by Shore & Chakrabarti (1976). The amount of labelling was determined by the A_{490} by using an absorption coefficient of $3.4 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Churchich, 1967). The preparations contained between 0.4 and 0.8 fluorescein molecule per equivalent of enzyme.

Vesicles. Unless otherwise stated, multilamellar vesicles were prepared by vortex-mixing lipid with enzyme in 100 mM-sodium/potassium phosphate buffer, pH 7.4, for 15 min as described by Bangham *et al.* (1965). To prepare unilamellar vesicles this step was followed by sonication for 1–2 h in a bath-type sonicator as described previously (Papahadjopoulos, 1970). All procedures were carried out under nitrogen and at 8°C . Multilamellar vesicles were separated by centrifuging at $40000g_{\text{av}}$ for 40 min, and were washed repeatedly with buffer. Unilamellar vesicles were separated on a column (80 cm $\times$ 2 cm) of Sephadex G-200, equilibrated with 100 mM-sodium/potassium phosphate buffer at pH 7.4 and pretreated with $20 \mu\text{mol}$ of 1:1 phosphatidylserine/phosphatidylcholine unilamellar vesicles, which saturated the phospholipid-binding sites on the Sephadex G-200 column. The position of the vesicle fractions was determined by adding a trace of di[^3H]palmitoyl phosphatidylcholine to the vesicle mix and counting radioactivity in the eluate as described below.

The volume of the internal compartment of large multilamellar vesicles was estimated by preparing the vesicles, as described above, in medium containing [^{14}C]glucose, and measuring the radioactivity associated with vesicle-pellet and supernatant fractions after separation.

Assays. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. In order to estimate the protein in vesicle fractions, the cloudy lipid precipitate that formed during the development of colour was removed by centrifugation before absorbance measurement.

Enzyme activity was measured as described by Gregory *et al.* (1971). In order to estimate the activity of enzyme trapped inside the vesicles, samples were either treated with 0.2% Triton X-100 or rapidly frozen in liquid nitrogen and thawed before addition to the assay cuvette. Both methods give a similar result, and treatment with Triton was used routinely. Activities are expressed as units, where 1 unit is equivalent to that amount of enzyme that catalyses

the conversion of 1 μmol of substrate in 1 min at 25°C.

Radioactivity. Samples were added directly to Phase Combining System scintillation 'cocktail' and counted at 5°C in a Nuclear-Chicago mark I scintillation spectrometer.

Fluorescence polarization. Fluorescence polarization was measured in a Perkin-Elmer MPF-44 fluorescence spectrophotometer equipped with a thermostatically controlled cell holder and a Hitachi 018-0054 polarization accessory. Measurements were made with excitation at 490 nm and emission at 520 nm, and polarization was calculated by the equation:

$$P = (|| - G \perp) / (|| + G \perp)$$

where $||$ and $\perp$ represent the measured relative fluorescence with the emission polarizer vertical and horizontal respectively and using vertically polarized excitation; G represents a correction factor consisting of the ratio of vertically to horizontally polarized emission with excitation in the horizontal plane (Azumi & McGlynn, 1962). Each reading was corrected for background scatter caused by solvent or vesicles. The proportion of monomer and dimer at any malate dehydrogenase concentration on the dissociation curve was calculated by assuming Weber's addition law (Weber, 1952a,b, 1953):

$$P(\text{obs.}) = 0.28 \frac{[x]}{[\text{mMDH}]} + 0.205 \frac{[y]}{[\text{mMDH}]}$$

where $[x]$ is the dimer concentration, $[y]$ is the monomer concentration and $\left[x + \frac{y}{2}\right]$ is equal to the total enzyme concentration; mMDH is mitochondrial malate dehydrogenase. The maximum and minimum experimentally determined polarization values of 0.28 and 0.205 represent 100% dimer and 100% monomer respectively. The rotational relaxation times (P_h) were calculated from curves of temperature-viscosity versus polarization by the Perrin equation for polarized incident light (Weber, 1953):

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right) \left(1 + \frac{3r}{P_h}\right)$$

where P is the measured degree of polarization, P_0 is the limiting polarization at low temperature and r is the excited lifetime of the fluorophore. A value of 5 ns was taken for the fluorescence lifetime (Shore & Chakrabarti, 1976). This value has been shown to remain constant over the concentration range studied here (Shore & Chakrabarti, 1976), and, as there was no significant variation in fluorescence intensity with temperature, a constant lifetime of the excited state was assumed under all conditions studied (Churchich, 1967). The relaxation time of an

unhydrated spherical molecule of known molecular weight can be calculated from the Stokes equation:

$$\rho_0 = 3\eta V/RT$$

where the molar volume $V = M\bar{v}$ (where M is molecular weight and $\bar{v}$ is partial specific volume) and η is the viscosity of the solvent at temperature T . An approximate value of 0.71 for $\bar{v}$ was calculated from the amino acid composition of malate dehydrogenase (Gregory *et al.*, 1971).

Circular dichroism. C.d. spectra were measured with a Cary 61 spectropolarimeter with thermostatically controlled sample holder. Cells with path lengths of 0.5 mm and 10 cm were used for concentrated (3 μM) and dilute (5 nM) malate dehydrogenase samples respectively. An estimate of the α -helical content was obtained by comparison of the curves with those of Greenfield & Fasman (1969) by the equation:

$$\% \alpha\text{-helix} = \frac{[\theta]_{210\text{ nm}} - 4000}{33000 - 4000}$$

where $[\theta]_{210\text{ nm}}$ is the mean residue ellipticity of the protein at 210 nm. A comparative computer analysis of the structures was not undertaken because the adsorption of very small amounts of protein on to the walls of the cells in the case of the dilute enzyme could result in overestimation of the effective protein concentration.

Results

Association of malate dehydrogenase with phospholipid vesicles

The extent of association of malate dehydrogenase with phospholipid vesicles can be readily examined by forming unilamellar vesicles in the presence of radioactively labelled enzyme and separating the vesicles from free enzyme by column chromatography on Sephadex G-200. As shown in Fig. 1(a), less than 0.5% of the radioactivity of bovine mitochondrial malate dehydrogenase (1.1 mg/ml) became associated with 1:1 phosphatidylserine/phosphatidylcholine vesicles. In similar experiments with multilamellar vesicles, the amount of radioactivity associated with the vesicles paralleled that of protein (results not shown). Also, with unlabelled enzyme, the same amount of unlabelled protein became associated with the vesicles, demonstrating that the labelling did not introduce artifacts (results not shown).

The amount of enzyme associated with vesicles, as measured by the enzyme activity released by Triton X-100, depended on the net charge of the phospholipids as shown in Fig. 1(b). The amount of enzyme released was approximately equal to the amount determined by radioactivity (Fig. 1a), indicating that there was little if any binding of enzyme to the

Fig. 1. Association of ^{14}C -labelled bovine malate dehydrogenase with unilamellar lipid vesicles

Unilamellar vesicles were prepared in 1 ml of 100 mM-sodium/potassium phosphate buffer, pH 7.4, with 1.1 mg (a) or 1–2 mg (b) of ^{14}C -labelled bovine malate dehydrogenase, 10 μmol of phospholipid and a trace of $\text{di}[^3\text{H}]\text{palmitoyl}$ phosphatidylcholine as described in the Experimental section. The samples were applied to a column (80 cm $\times$ 2 cm) of Sephadex G-200 and fractions measured for radioactivity (a) or enzyme activity released after treatment with Triton X-100 (b). The lipids used were 1:1 phosphatidylserine/phosphatidylcholine (a) and stearylamine or phosphatidylserine plus phosphatidylcholine as indicated (b). In (b) the proportionate charge is equal to the vesicle content of charged lipid on a w/w basis. In (b) bars denote s.d. calculated from at least three separate experiments for each point. (a) ●, ^3H (vesicles); ○, ^{14}C (malate dehydrogenase).

phospholipid. Further, the amount of enzyme associated gives a measure of the entrapped aqueous volume if there is no binding of enzyme to phospholipid. The volume and its variation with net charge of the phospholipid is close to that demonstrated by others (Papahadjopoulos & Kimelberg, 1973). In similar experiments not shown here we also found that the enzyme did not bind to a crude extract of mitochondrial phospholipids.

Effect of phospholipids on the stability of dissociated malate dehydrogenase

Malate dehydrogenase is a dimer and, although no stable association of enzyme with phospholipid was observed, it was possible that the monomer would interact. As a preliminary to such studies, conditions under which the enzyme dissociates were examined and the effect of phospholipids on the stability of dissociated enzyme was determined.

The state of dissociation of mitochondrial malate dehydrogenase can be determined by measuring the polarization of fluorescence of fluorescein-labelled enzyme. This has previously been demonstrated by Shore & Chakrabarti (1976), and the interpretation of their data has been supported by gel-filtration and sedimentation studies (Bleile *et al.*, 1977). All techniques demonstrated a concentration- and pH-dependent dissociation of porcine mitochondrial malate dehydrogenase. From studies using fluorescence depolarization we have calculated the dissociation constant (K_d) for the porcine enzyme to be 0.24 mM in 100 mM-sodium/potassium phosphate

buffer at pH 7.0 and 21°C. Neither the porcine enzyme in the presence of 20 mM- NAD^+ or 5 mM- NADH nor the bovine enzyme dissociated above 0.01 μM , at which concentration the porcine enzyme was fully dissociated. Furthermore, if coenzyme was added to the dissociated fluorescein-labelled porcine enzyme, it caused an immediate increase in polarization consistent with dimerization.

The native dimer of porcine mitochondrial malate dehydrogenase was stable to thermal inactivation, but the dissociated enzyme was inactivated at 31°C with typical first-order kinetics (Fig. 2a). The rate constant for inactivation of 0.15 μM -enzyme at pH 7.0 was $0.18 \pm 0.04 \text{ ms}^{-1}$ (4) (mean $\pm$ s.d. with the number of experiments given in parentheses). When dilute enzyme was incubated with 10 mM- NAD^+ , inactivation was inhibited by more than 95%. The k_{inact} was proportional to $\log$ (enzyme concentration) within the range of the enzyme dissociation (inset of Fig. 2a). These experiments were carried out in silicone-coated glassware with a minimal air/water surface area, so that surface inactivation effects would be negligible. The results suggest that enzyme subunits are less stable than the native dimer. The pH-dependence of inactivation is shown in Fig. 2(b). Whereas the native dimer at 7 μM was stable over the pH range 6.0–9.0 (results not shown), the dilute enzyme was very rapidly inactivated at both extremes of pH and had an optimum stability at pH 7.4.

Fig. 2(c) shows the effects of phospholipids on inactivation. When unilamellar vesicles containing 1:1 phosphatidylserine/phosphatidylcholine or 1:9

Fig. 2. Dependence of inactivation of porcine malate dehydrogenase on enzyme concentration, coenzymes, pH and phospholipid. Porcine malate dehydrogenase was incubated at 31°C in 100mM-sodium/potassium phosphate buffer, pH 7.0, and samples were removed at 10min intervals and assayed for enzyme activity. Results are expressed as the log of the percentage of enzyme activity remaining after each time interval plotted against time. In all cases, the data are the means of at least three experiments. In (a) the effect of enzyme concentration and coenzymes on inactivation was examined. The inset gives the dependence of $10^{-2} \times k_{\text{inact}}$ (s $^{-1}$) on enzyme concentration (μM). ●, 0.14 μM -Enzyme (10 $\mu\text{g}/\text{ml}$); ○, 28 μM -enzyme (2mg/ml); △, 0.14 μM -enzyme plus 10mM-NAD $^{+}$ or 0.5mM-NADH. In (b) the effect of pH on inactivation was determined at an enzyme concentration of 0.14 μM . The inset gives the dependence of $10^2 \times t_{1/2}$ (min) of inactivation on pH. □, pH 6.0; ●, pH 7.0; ○, pH 7.4; △, pH 8.0; ▲, pH 9.0. In (c) the effect of phospholipid on inactivation was examined. In each case the malate dehydrogenase was incubated at 0.14 μM . Inactivation constants with standard deviations are given in the text. ●, Control; □, 7 μmol of 1:1 phosphatidylserine/phosphatidylcholine unilamellar vesicles/ml; ○, 7 μmol of phosphatidylcholine unilamellar vesicles/ml; △, 7 μmol of 1:9 stearylamine/phosphatidylcholine unilamellar vesicles/ml.

stearylamine/phosphatidylcholine were added to malate dehydrogenase, the rate of inactivation was decreased by over 7-fold [k_{inact} of $27.5 \pm 5.5 \mu\text{s}^{-1}$ (3) compared with $0.18 \pm 0.04 \text{ms}^{-1}$ (4)]. Neutral phosphatidylcholine vesicles did not significantly affect inactivation. The increased stability of the dilute porcine enzyme in the presence of phospholipid vesicles suggests an association of enzyme monomer and phospholipid. Previously Callahan & Kosicki (1967) had found that a diverse group of lipids stabilized the enzyme, but the state of dissociation was not determined.

Inactivation by *p*-chloromercuribenzoate

The ability of phospholipids to protect the enzyme from inactivation by thiol-group-blocking reagents was also examined. Gregory *et al.* (1971) and Bleile *et al.* (1977) have demonstrated that there is a thiol group near to or at the active site of mitochondrial malate dehydrogenase which becomes exposed and accessible to thiol-group-blocking reagents when the enzyme dissociates. Titration of this group was shown by them to be paralleled by enzyme inactivation.

The effects of coenzymes and phospholipid vesicles on the inactivation of dilute porcine malate dehydro-

genase by *p*-chloromercuribenzoate are shown in Fig. 3. Under all conditions the inactivation followed pseudo-first-order kinetics after an initial lag phase. Both NADH and NAD $^{+}$ protected the enzyme from inactivation, in agreement with the observations that coenzyme binding prevents dissociation, thereby causing the active-site thiol group to remain buried. The inactivation by *p*-chloromercuribenzoate was unaffected when the enzyme was incubated with phosphatidylcholine unilamellar vesicles, but, when unilamellar vesicles consisting of 1:1 phosphatidylserine/phosphatidylcholine or 1:9 stearylamine/phosphatidylcholine were incubated with dilute enzyme, inactivation by *p*-chloromercuribenzoate was inhibited. The 5-fold decrease in k_{inact} for *p*-chloromercuribenzoate inactivation caused by incubation with the vesicles was about the same as that for the thermal inactivation (see Fig. 2c).

Binding of malate dehydrogenase to multilamellar phospholipid vesicles

In a more direct approach to the study of binding of subunits to phospholipids, ^{14}C -labelled porcine malate dehydrogenase was mixed with phospholipid, multilamellar vesicles were formed, and bound and

Table 1. Comparison of the association of concentrated and dilute ^{14}C -labelled porcine malate dehydrogenase and ^{14}C -glucose with multilamellar phospholipid vesicles. Multilamellar vesicles were prepared as described previously by vortex-mixing $10\text{ }\mu\text{mol}$ of the indicated lipid in 2 ml of 100 mM -sodium/potassium phosphate buffer, $\text{pH } 7.0$, containing ^{14}C -labelled malate dehydrogenase at the concentration indicated or ^{14}C -glucose. The vesicles were separated from free enzyme by centrifugation, and the protein, ^{14}C -radioactivity and enzyme activity contained in all fractions were measured as described in the Experimental section. Entrapped enzyme was released from a portion of the vesicle pellet with 0.2% Triton X-100. The radioactivity of active enzyme was calculated from the specific radioactivity, and units bound and the difference between this and the total bound was the radioactivity of inactive enzyme. The data shown were from a single experiment and the apparent vesicle volumes are given as means $\pm$ s.d. for several experiments as indicated. The apparent vesicle volume was the volume that would be occupied by the portion of label that became associated with the vesicle fraction if no interaction occurred. Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; SA, stearylamine.

Vesicles ...	Mitochondrial malate dehydrogenase										^[14C] - Glucose PS/PC (c.p.m.)
	15 μM (0.81 mg/ml)					0.1 μM (10 μg/ml)					
	PS/PC		PC		SA/PC	PS/PC		PC		SA/PC	
	(c.p.m.)	(units)	(c.p.m.)	(units)		(c.p.m.)	(units)	(c.p.m.)	(units)		
Fraction											
Total	0.39 × 10 ⁶	2740	0.9 × 10 ⁶	2300	1.1 × 10 ⁶	1.4 × 10 ⁴	10	1.2 × 10 ⁴	10	1.2 × 10 ⁴	3.3 × 10 ⁶
Free											
(supernatant)	0.37 × 10 ⁶	2703	0.8 × 10 ⁶	2240	0.85 × 10 ⁶	1.08 × 10 ⁴	7.7	0.8 × 10 ⁴	6.6	0.71 × 10 ⁴	3.4 × 10 ⁶
Vesicle-bound	0.012 × 10 ⁶	18.5	0.1 × 10 ⁵	13	0.8 × 10 ⁵	0.23 × 10 ⁴	15	0.09 × 10 ⁴	0.41	0.18 × 10 ⁴	0.023 × 10 ⁶
Active	0.025 × 10 ⁵	18.5	0.46 × 10 ⁴	13	0.58 × 10 ⁵	0.07 × 10 ⁴	15	0.05 × 10 ⁴	0.41	0.04 × 10 ⁴	—
Inactive	0.095 × 10 ⁵	0	0.54 × 10 ⁴	0	0.22 × 10 ⁵	0.16 × 10 ⁴	0	0.04 × 10 ⁴	0	0.14 × 10 ⁴	—
Apparent vesicle volume (μl)	14.1 ± 2.6 (4)		11.1 ± 3.1 (3)		13.2 ± 4.1 (3)	140 ± 15.6 (4)		79 ± 17 (3)		65 ± 14 (2)	12

Fig. 3. Inactivation of porcine malate dehydrogenase by *p*-chloromercuribenzoate as a function of time

Porcine malate dehydrogenase at $0.14 \mu\text{M}$ was incubated in 100 mM-sodium/potassium phosphate buffer, pH 7.0, at 4°C with 0.1 mM *p*-chloromercuribenzoate. Samples from the reaction mixture were removed at the indicated times and assayed for enzyme activity. The inactivation is represented as the log of the ratio V_i/V_c (velocity of inhibited sample to velocity of a control sample). The data are the means for three experiments for each curve. ●, Control, no further additions; ▲, $7 \mu\text{mol}$ of phosphatidylcholine unilamellar vesicles/ml; ○, $7 \mu\text{mol}$ of 1:1 phosphatidylserine/phosphatidylcholine unilamellar vesicles/ml; □, $7 \mu\text{mol}$ of 1:9 stearylamine/phosphatidylcholine unilamellar vesicles/ml; ■, 10 mM-NAD^+ or 2 mM-NADH .

free enzyme were separated by centrifugation (Table 1). The fraction of enzymically active ^{14}C -labelled malate dehydrogenase associated with the vesicle pellet, as estimated by the apparent aqueous volume, at a concentration ($15 \mu\text{M}$) at which only dimer molecules are expected to be present, correlates well with the aqueous volume of the vesicles, as measured by ^{14}C glucose entrapment. This is similar to the results with the bovine enzyme and unilamellar vesicles shown in Figs. 1(a) and 1(b), and it can be concluded that the associated active enzyme was not bound to phospholipid but was simply trapped inside the vesicles during their formation. When multilamellar vesicles were prepared in medium containing $0.1 \mu\text{M}$ porcine malate dehydrogenase, the ratio of vesicle-associated active enzyme to supernatant active enzyme was increased approx. 10-fold. If the association were simply due to physical entrapment, the apparent vesicle volume would be about $12 \mu\text{l}$ rather than the equivalent of $65\text{--}140 \mu\text{l}$ found with different types of vesicles. Therefore the active malate dehydrogenase that became encapsulated must have interacted with the phospholipid during vesicle formation.

In experiments not shown here the interaction of bovine malate dehydrogenase, at low enzyme concentration, with multilamellar vesicles was also investigated, as described in Table 1 for the dissociated porcine enzyme. Although the bovine enzyme retained its dimeric form at high dilution, a portion of the enzyme equivalent to about 60% of the porcine enzyme became bound to the vesicles. Under such circumstances it is possible that the phospholipid

Table 2. Comparison of the binding of ^{14}C -labelled porcine and bovine malate dehydrogenases to preformed phospholipid vesicles

Conditions and measurements were the same as described in Table 1. Phosphatidylserine/phosphatidylcholine (1:1, w/w, $10 \mu\text{mol}$) was vortex-mixed in 2 ml of 100 mM-sodium/potassium phosphate buffer, pH 7.0. Enzyme was added to the multilamellar vesicles and the mixture incubated at 21°C for a further 15 min. The vesicles were then separated from free enzyme by centrifugation and each fraction was assayed for radioactivity and enzyme activity. Entrapped enzyme was released from the vesicle pellet by the addition of 0.2% Triton X-100 before assaying for enzyme activity. The pellets were not washed in the experiments with low enzyme concentration but were with the concentrated porcine enzyme, and corrections were made for the free enzyme that would be associated with the pellet in the former cases. Results for amount of enzyme are from one experiment, the values in the top two rows being c.p.m. and those in the bottom three being units of enzyme activity. Percentages are mean values $\pm$ s.d.

Fraction	Malate dehydrogenase					
	Porcine ($0.1 \mu\text{M}$; $9 \mu\text{g/ml}$)		Bovine ($0.1 \mu\text{M}$; $9 \mu\text{g/ml}$)		Porcine ($7 \mu\text{M}$; 0.5 mg/ml)	
	Amount	%	Amount	%	Amount	%
Free enzyme (c.p.m.)	18040	91.9 ± 4 (3)	17980	96.4 ± 5.2 (3)	17000	100
Vesicle-associated enzyme (c.p.m.)	1360	8.1 ± 2.6 (3)	550	3.6 ± 1.6 (3)	20	0
Free activity (units)	9.6	95.2 ± 4.8 (3)	9	100	450	100
Vesicle-associated activity; no Triton (units)	0.15	2.1 ± 0.8 (3)	0	0	0	0
Vesicle-associated activity; +Triton (units)	0.59	4.8 ± 1.7 (3)	0	0	0	0

membrane surface may induce a dissociation of the bovine enzyme (see the Discussion section).

Binding of malate dehydrogenase to preformed vesicles

A comparison of the binding of porcine and bovine malate dehydrogenase to preformed 1:1 phosphatidylserine/phosphatidylcholine vesicles is shown in Table 2. At the dilute enzyme concentrations, the porcine malate dehydrogenase was about 50% dissociated, whereas the dissociation of the bovine malate dehydrogenase was negligible. Active enzyme became associated with the vesicles only when the dilute porcine enzyme was used. This enzyme could be measured only after treatment of vesicles with Triton X-100. Changes in pH over the range of 7.0–8.0 did not affect the binding of active enzyme.

Effect of phospholipid vesicles on the polarization of fluorescence of fluorescein-labelled malate dehydrogenase

The rate of thermal inactivation of 0.15 μM porcine malate dehydrogenase was decreased 7-fold when 5 μmol of 1:1 phosphatidylserine/phosphatidylcholine unilamellar vesicles/ml was incubated with the enzyme (Fig. 2c); however, less than 10% of active enzyme remained associated with multilamellar vesicles after centrifugation (Table 2). Although this discrepancy could be associated with the difference in the exposed surface area (Papahadjopoulos & Kimelberg, 1973) of unilamellar vesicles (used for binding experiments), it is more likely that the enhanced enzyme stability caused by phospholipid vesicles was due to transitory associations of malate

dehydrogenase with the phospholipid bilayer. If this were the case, then the data shown in Tables 1 and 2 may underestimate the enzyme-vesicle associations, because the methods used for preparation and/or separation would disturb the dynamic equilibrium that exists when vesicles and enzyme are in solution. To investigate this possibility and to examine further the nature of the interaction, we have studied the influence of phospholipid vesicles on the polarization of fluorescence of fluorescein-labelled porcine and bovine malate dehydrogenase.

Fig. 4a shows a Perrin plot of fluorescein-labelled concentrated porcine malate dehydrogenase, and dilute porcine malate dehydrogenase with and without 1:1 phosphatidylserine/phosphatidylcholine unilamellar phospholipid vesicles. Linear relationships were observed and the plot of the dilute (monomer) malate dehydrogenase was biphasic, showing an abrupt decrease in polarization between 19 and 23°C. This temperature-induced biphasic response was eliminated when the dilute enzyme was incubated with phospholipid vesicles. The addition of vesicles to concentrated porcine malate dehydrogenase had no effect on depolarization. There was no significant loss of enzyme activity during the course of any of these experiments. In addition to preventing the biphasic temperature response of dilute enzyme, polarization values were consistently greater in the presence of phospholipid vesicles. Both of these observations suggest an interaction between the vesicles and fluorescein-labelled porcine malate dehydrogenase monomer. The change in the intercept on the $1/P$ axis, i.e. the limiting polarization, observed when the vesicles were present, is usually indicative

Fig. 4. Perrin plots of fluorescein-labelled porcine (a) or bovine (b) malate dehydrogenases recorded over a temperature range of 5–30°C

Polarization measurements in 100mM-sodium/potassium phosphate buffer, pH 7.0, and calculations were as described in the Experimental section. The solid lines are regression curves and each experimental point is the average of at least three experiments. In all cases control experiments lacking enzyme only were run in parallel with test cuvettes, so that background scatter could be subtracted for each reading. The signal-to-noise ratio was sufficiently high to give a standard error of less than $\pm 5\%$ at the lowest concentrations studied. Each enzyme equivalent contained 0.78 (porcine) or 0.80 (bovine) fluorescein group. η is the viscosity of the sample. (a) ●, 0.08 μM -Malate dehydrogenase; ○, 24 μM -malate dehydrogenase; ▲, 0.08 μM -malate dehydrogenase plus 2.3 μmol of 1:1 phosphatidylserine/phosphatidylcholine unilamellar vesicles/ml. (b) ●, 0.078 μM -Malate dehydrogenase; ○, 19 μM -malate dehydrogenase; ▲, 0.078 μM -malate dehydrogenase plus 2.3 μmol of 1:1 phosphatidylserine/phosphatidylcholine unilamellar vesicles/ml.

Table 3. Relaxation time (P_h), relaxation ratio (P_h/ρ_0) and limiting polarization (P_0) of concentrated and dilute malate dehydrogenase in the presence and absence of 1:1 phosphatidylserine/phosphatidylcholine unilamellar vesicles.

Values were determined from Perrin plots of Fig. 4(b) as described in the Experimental section. A mol.wt. of 70000 for enzyme dimer and 35000 for monomer was assumed. ρ_0 is the theoretical relaxation time for an unhydrated sphere calculated by using the Stokes equation (see the Experimental section for equations).

Enzyme	P_h (ns)		ρ_0 (ns)		P_h/ρ_0		P_0
	10°C	22°C	10°C	22°C	10°C	22°C	
Porcine							
Concentrated (24 μ M)	53.0	37.3	83.0	58.0	0.64	0.64	0.281
Dilute (0.08 μ M)	16.0	9.4	41.0	29.0	0.40	0.32	0.295* (0.287)*
Dilute plus vesicles	13.4	9.3	41.0	29.0	0.32	0.32	0.383
Bovine							
Concentrated (19 μ M)	42.1	29.6	83.0	58.0	0.51	0.51	0.331
Dilute (0.08 μ M)	31.0	21.6	72.0	50.6	0.42	0.42	0.332
Dilute plus vesicles	33.9	24.0	72.0	50.6	0.47	0.47	0.335

* The two values are extrapolations of the two linear portions of the plot for dilute porcine malate dehydrogenase in Fig. 4(a).

Fig. 5. C.d. spectra of porcine malate dehydrogenase

Measurements were as described in the Experimental section with enzyme in 100mM-sodium/potassium phosphate buffer, pH7.0. ●, 30 μ M-Enzyme with a cell of path length 5mm; ○, 43 nM-enzyme in a cell of path length 10cm. Spectra were calculated from the recordings of three separate experiments in each case.

fluorescein-labelled bovine malate dehydrogenase. No extensive dissociation of this enzyme occurred, and the response of the diluted enzyme was not biphasic. The slopes of the curves for the bovine enzyme were all fairly similar compared with the large differences noted when the porcine enzyme was used, and vesicles had very little effect on the polarization, with no change in the limiting polarization.

Table 3 shows the relaxation times and ratios and limiting polarization values for malate dehydrogenase calculated from the Perrin plots. The two different relaxation ratio values (P_h/ρ_0) (10 and 22°C) shown for the porcine malate dehydrogenase monomer correspond to the polarization values above and below the transition in the Perrin plot. It should be noted that only experimental relaxation times (P_h) calculated by using data with the same intercept on the $1/P$ axis can be directly compared because a change in P_0 (i.e. limiting polarization at zero absolute temperature and viscosity when no molecular rotation occurs) results from a change in shape or fluorescent-ligand attachment (Weber, 1952b, 1953).

Circular dichroism

The exposure of new groups after enzyme dissociation (Fig. 3) and the biphasic Perrin plot of dissociated enzyme suggests that the conformation of the monomer is different in the dissociated and undissociated forms of the enzyme. To investigate this, c.d. spectra of native (3 μ M) and dissociated (4 nM) porcine malate dehydrogenase were measured in the 200–250 nm range as shown in Fig. 5. From the results, rough approximations of the α -helical content were calculated by the methods of Greenfield & Fasman (1969) and gave values of $50 \pm 2.4\%$ (3) for the native malate dehydrogenase and $46 \pm 4.1\%$

of a change in shape or change in environment of the fluorescent ligand (see the Discussion section).

Curves analogous to those of porcine malate dehydrogenase in Fig. 4(a) are shown in Fig. 4(b) for

(3) for the subunits. The spectra are quite similar, so that only small changes in polypeptide conformation may occur as a result of dissociation. A reproducible feature was the loss of the positive inflection at 212.5 nm in the spectrum of the dissociated porcine enzyme. This change was not observed when the spectra of the dilute and concentrated bovine enzymes were compared under identical conditions (results not shown). In the case of the bovine enzyme both spectra were similar to that of the concentrated porcine sample shown in Fig. 5. The change in the spectrum of the dilute porcine enzyme, which may result from dissociation into subunits, could represent an increase in β -sheet structure, which has been shown to have a negative maximum between 210 and 220 nm (Greenfield & Fasman, 1969), but other interpretations are possible: for example, a change in this low magnitude could also result from reorientation of side-chain chromophores (Filippi *et al.*, 1976).

Discussion

The results demonstrate that enzymically active malate dehydrogenase dimer does not bind to phospholipid vesicles. From the amount associated, it is concluded that the enzyme is encapsulated in the aqueous volume of uni- and multi-lamellar vesicles. In contrast, the enzyme's subunits can interact with phospholipid, as shown by binding measurements, the enhanced stability of the enzyme to inactivation by heat and thiol-group-blocking reagents in the presence of vesicles and the effect of vesicles on the characteristics of the Perrin plot of dissociated enzyme.

Although there is no direct evidence, it is likely the phospholipid vesicles interact with enzyme subunits, in part at least, at the subunit-subunit binding site. Blocking of this site in the dimer prevents inactivation by both heat and thiol-group reagents, as shown by our results and those of Gregory *et al.* (1971) and Bleile *et al.* (1977). Bleile *et al.* (1977) suggested that the site of subunit-subunit binding may be hydrophobic, and, if so, the exposure of this surface to a polar environment would be expected to produce a less-stable structure. The phospholipid vesicles would enhance subunit stability by providing a hydrophobic environment for the subunit surface. Binding involving this site would obviously be specific for the dissociated enzyme. The results presented in the Perrin plot support this interpretation. Because of the change in limiting polarization when the porcine subunits were incubated with vesicles (Fig. 4a), the data cannot be used to calculate binding constants (Johnson & McIntosh, 1976; Levinson *et al.*, 1976). However, a change of limiting polarization is usually interpreted as a change in the

environment of the fluorescent ligand (Weber, 1953). In this case the change caused by the phospholipid could be due to either direct interaction with ligand or an enzyme conformational change. In either case the changes in slope and intercept of the curve are consistent with a subunit-vesicle interaction, which is not apparent when the native dimer is mixed with phospholipid.

It should be noted that if the interactions between subunit and vesicles are partially hydrophobic, which seems probable, then the malate dehydrogenase subunit may undergo changes analogous to those described for proteins of the amphipathic helix type when they interact with phospholipids. The high content of α -helix, flexible structure and high average hydrophobicity [47% from the amino acid composition (Gregory *et al.*, 1971)] would favour such an explanation.

Although subunit-vesicle association may involve hydrophobic interactions, the observation that only vesicles with a net charge prevented subunit inactivation indicates that ionic and pH effects are probably important also. The binding of monomer to phosphatidylcholine vesicles must be of a different nature as it does not stabilize the enzyme. Since malate dehydrogenase is close to its isoelectric point at neutral pH (Strasberg & Freeman, 1975), interaction with charged vesicles could result from the induced charge caused by the pH differential at the bilayer interface (Fromherz & Masters, 1974; Peters & Fromherz, 1975). Peters & Fromherz (1975) demonstrated a binding of porcine malate dehydrogenase to charged fatty acid monolayers. By measuring changes in surface pressure when malate dehydrogenase was injected into the subphase below phospholipid monolayers, we have also found that the enzyme subunit binds to lipid under conditions where hydrophobic penetration is possible (K. A. Webster, K. B. Freeman & S. Ohki, unpublished observations). In the experiments of Peters & Fromherz (1975) the enzyme concentration was such that the enzyme would be appreciably dissociated under their conditions and the binding was irreversible and appeared to cause enzyme inactivation. At the very low subphase buffer concentration used to maximize effects, with changes at the monolayer/subphase boundary as large as 4 pH units (Fromherz & Masters, 1974), it is possible that the enzyme subunits that are labile to extreme pH would be inactivated during binding to the monolayer. For the experiments described here the use of a higher buffer concentration appears to prevent enzyme inactivation but still allows enzyme subunits to interact with charged vesicular membranes.

In addition to the possible exposure of a hydrophobic surface, enzyme dissociation may be accompanied by a small conformational change, as shown by the change in the c.d. spectrum and the biphasic

Perrin plot. The latter, which results in a sharp change in the subunit relaxation ratio at 21°C (Table 2), can be interpreted as a temperature-induced increase in molecular flexibility or an increase in the axial ratio (Johnson & McIntosh, 1976). Since this change does not occur when dilute bovine malate dehydrogenase is used under identical conditions, or when concentrated porcine enzyme is used, it is probable that this phenomenon is specific for the subunit. Supposing that the transition resulted in a less-stable structure at the higher temperature, then the inhibition of this transition and the increase in enzyme stability, which are both caused by incubation with vesicles, may be associated events.

The properties and interactions of the subunits of mitochondrial malate dehydrogenase are unique insofar as they distinguish the dissociated enzyme from the native form and from cytosolic malate dehydrogenase. It has previously been shown that the cytosolic enzyme does not dissociate, even under conditions of extreme dilution and pH (Bleile *et al.*, 1977). The interaction of the subunit with phospholipid and the flexible conformation of the subunit may reflect properties of the dissociated mitochondrial isoenzyme that determine its transport and sequestration inside mitochondria. Two essential requirements for this transport are a means of determining specificity and a mechanism to overcome the thermodynamic problem of transporting a large hydrophilic protein across the hydrophobic interior of the mitochondrial membranes. We propose a model involving the diffusion of subunits across the membranes followed by dimerization inside the mitochondrial matrix. Diffusion of the subunits is possible because of the properties discussed above, allowing the subunits to interact with the membrane phospholipids and then to refold so that hydrophobic residues are exposed to the hydrophobic membrane interior. This amphipathic property of the subunit is essential for diffusion within and without the membrane. Refolding for membrane penetration may be initiated by the primary interaction of the membrane phospholipid with a hydrophobic region of the subunit. Dimerization may result from interaction with coenzymes and substrates on the matrix side of the mitochondrial inner membrane or as a result of the pH differential across the mitochondrial membranes (Mitchell & Moyle, 1969). Dimerization will cause the enzyme to become fixed inside the matrix compartment. More direct specificity could arise if it were demonstrated that there were target sites on the mitochondrial membranes (Blobel & Dobberstein, 1975*a,b*) that would bind enzyme subunits but not the native enzyme.

Although the release of enzyme subunits from ribosomes has not been definitely shown, it should be noted that the cytosolic amounts of malate

dehydrogenase and other mitochondrial matrix enzymes *in situ* will probably be such that the molecules will remain as subunits until compartmentation inside the mitochondrion. Therefore the addition of purified concentrated enzymes to mitochondria to study uptake may lead to ambiguous results, depending on the incubation conditions and their effect on the enzyme dissociation (Hallermayer *et al.*, 1977; Harmey *et al.*, 1977; Marra *et al.*, 1977, 1978).

The translocation of enzyme subunits across the mitochondrial membranes in the manner proposed above requires neither specific cellular sites of synthesis, including vectorial discharge from ribosomes into mitochondria (Kellems *et al.*, 1974; Blobel & Dobberstein, 1975*a,b*; Shore & Tata, 1977), nor post-transcriptional enzyme modification (Blobel & Dobberstein, 1975*a,b*; Shore & Harris, 1977), other than polymerization in the mitochondrial matrix. Recent reports suggest that mitochondrial matrix enzymes or their precursors are free in the cytosol before uptake (Marra *et al.*, 1977, 1978; Hallermayer *et al.*, 1977; Harmey *et al.*, 1977). The properties that would allow mitochondria to distinguish between different proteins in the cytosol is unclear, and in this respect the region of subunit-subunit contact of matrix enzymes may be of importance.

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