Hydrophobic Interaction between the Monomer of Mitochondrial Malate Dehydrogenase and Phospholipid Membranes

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Porcine mitochondrial malate dehydrogenase (EC 1.1.1.37) dissociates into subunits on dilution. The enzyme monomer caused large increases in the surface pressure of monolayers of 1:1 phosphatidylserine/phosphatidylcholine at air/water and oil/water interfaces. The monomer increased the permeability of phospholipid vesicles to $^{22}\text{Na}^+$. Both effects were significantly greater than the corresponding effects of ribonuclease A, cytochrome *c* and the dimeric form of malate dehydrogenase. Changes in the circular-dichroism spectra of the enzyme indicated that conformational changes may be associated with dimer formation or when monomer interacts with lysophosphatidylcholine. Similar interactions to those described may occur *in situ* when mitochondrial malate dehydrogenase is transported to the mitochondrial matrix from its site of synthesis on cytosolic ribosomes.

Most of the proteins contained in the mitochondrial matrix are synthesized in the cytoplasm and transferred into the matrix across the two mitochondrial membranes (Schatz & Mason, 1974). Once inside the matrix these proteins, including the citric-acid-cycle enzymes, may be free or loosely bound to the inner surface of the inner membrane (Julliard & Gautheron, 1972; Waksman & Rendon, 1974; Erenk & Friedman, 1978). At present there is no direct evidence for transport pores, vectorial discharge or post-transcriptional modification concerned with the transport of the soluble matrix proteins (Godinot & Lardy, 1973; Hallermayer *et al.*, 1977; Harmey *et al.*, 1977; Marra *et al.*, 1978). This is in contrast with some intrinsic membrane proteins, which have been shown to be synthesized in precursor forms (Ries *et al.*, 1978; Maccacchini *et al.*, 1979; Coté *et al.*, 1979). By using mitochondrial malate dehydrogenase (EC 1.1.1.37) from porcine heart and model phospholipid membranes our studies have been directed towards determining how transport of this enzyme into the matrix occurs (Webster *et al.*, 1979). We have shown that mitochondrial malate dehydrogenase monomer can associate with phospholipid vesicles, whereas the native dimer does not. From this observation together with some physical properties of malate de-

hydrogenase monomer we suggested that transport may occur by diffusion of the monomer across the mitochondrial membrane followed by fixation in the matrix as the dimer. In the present paper we give further evidence to support this hypothesis by showing that the enzyme monomer can penetrate the hydrophobic region of model phospholipid membranes and that conformational changes accompany subunit-subunit and enzyme-phospholipid interactions.

Experimental

Materials

Phosphatidylcholine and phosphatidylserine were purchased from Serdary Research Laboratories Inc., London, Ont., Canada, and stored at -20°C until use. Both were 98% pure as estimated by t.l.c. For membrane formation 1:1 mixtures of these phospholipids were used routinely, since this combination has a surface charge approximately equal to that of phospholipid in the mitochondrial inner membrane (Stoffel & Schiefer, 1968). Also, as found by Dodd (1973), the mixture was considerably more stable than mixtures containing cardiolipin. Mitochondrial malate dehydrogenase (porcine) was from Miles Seravac (lot 7067), Elkhart, IN, U.S.A., or from Boehringer Mannheim (Canada) (batch 1167138), St. Laurent, Que., Canada, and was of the highest

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specific activity available. Ribonuclease A was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. (4800 units/mg; lot RAF 53N523) and cytochrome *c* was from Sigma Chemical Co., St. Louis, MO, U.S.A. (horse heart; type III, lot 124C-7250). ^{22}Na (carrier-free as NaCl in water) was from New England Nuclear (Canada), Lachine, Que., Canada. Phase-combining system scintillation 'cocktail' was purchased from Amersham/Searle Corp., Arlington Heights, IL, U.S.A. Hexane (monolayer-film spreading solution) was Baker Instra-Analysed grade and hexadecane (used for the oil/water interphase), >99% pure by gas chromatography, was from Fluka, A.G., Hauppauge, NY, U.S.A. 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 involving the binding and removal of denatured enzyme by mixing with phospholipid as described previously (Webster *et al.*, 1979).

Surface-tension measurements. Surface-tension measurements of 1:1 phosphatidylserine/phosphatidylcholine monolayers at the air/water interface were made as previously described (Seimiya & Ohki, 1972) by using a circular Pyrex glass trough with constant surface area ($\approx 20\text{ cm}^2$) and a microscopic cover slip ($18\text{ mm} \times 8\text{ mm} \times 0.16\text{ mm}$) as a Wilhelmy plate. Measurements at the oil/water interface were made as described by Ohki *et al.* (1976) with the same trough and plate as used for measurement at the air/water interface. Monolayers were formed at the oil/water interface by spreading 1:1 phosphatidylserine/phosphatidylcholine in hexane at the air/water interface and then carefully applying oil (hexadecane) on to the formed air/water monolayer (Ohki *et al.*, 1976). Hexane was used as the spreading agent for monolayers at both interfaces.

The proteins were dissolved in 100 mM-sodium/potassium phosphate buffer, pH 7.4, usually to 4 mg/ml, and the specified amount was injected into the subphase below the monolayer at zero time. The subphase was 100 mM-sodium/potassium phosphate buffer at pH 7.0 and 22°C and it was continuously stirred during the experiment, usually over a period of 60 min.

Permeability studies. The procedure used for the entrapment of $^{22}\text{NaCl}$ solution inside vesicles was similar to that described by Kimelberg & Papahadjopoulos (1971). Unilamellar phospholipid vesicles containing 1:1 phosphatidylserine/phosphatidylcholine were prepared by sonicating in a bath-type sonicator in radioactive exchange medium containing 10 mM-NaCl/20 mM-sodium/potassium phosphate, pH 7.0, and 5–10 μCi of $^{22}\text{NaCl}$ /ml at 22°C. En-

capsulated medium was separated from free medium by passing the vesicles through a column ($15\text{ cm} \times 1.5\text{ cm}$) of Sephadex G-50 with unlabelled exchange medium. The vesicle fractions were pooled and samples were taken for radioactivity determination. Portions of 0.5 ml were then pipetted into washed dialysis bags ($1.0\text{ cm} \times 10\text{ cm}$), additions of protein or buffer were made to the dialysis bag and any free $^{22}\text{NaCl}$ was removed by a preliminary 30 min dialysis against non-radioactive exchange medium.

Diffusion of the isotope was determined by incubating the dialysis sacs in duplicate stoppered tubes containing 12 ml of the exchange medium at 22°C. The incubations were stirred continuously. At intervals of 1 h the dialysis sacs were transferred to tubes containing fresh exchange medium. Radioactivity was determined in the 12 ml of diffusate sample and finally in the contents of the dialysis sacs. Self-diffusion rates were calculated from the amount of ^{22}Na in the diffusate during the first 2 h although diffusion rates were constant over periods up to 4 h.

The self-diffusion rate is expressed as a percentage of the ions present inside the vesicles at zero time/hour per μmol of lipid. The permeability coefficients in cm^2/s were calculated on the basis of an area of $1800\text{ cm}^2/\mu\text{mol}$ of lipid (Kimelberg & Papahadjopoulos, 1971) and a $^{22}\text{Na}^+$ concentration difference of $30\text{ } \mu\text{equiv.}/\text{cm}^3$.

Circular dichroism. Spectra were measured with a Cary 61 spectropolarimeter as described previously (Webster *et al.*, 1979). Before application to the sample cell the enzyme was diluted and addition of coenzyme or phospholipid was made. Protein and enzyme activity were determined from portions before and after the measurement of spectra. To study the interaction of enzyme with lysophosphatidylcholine appropriate amounts of each were mixed on ice. The mixture was then warmed at 27°C and agitated until the lipid suspension cleared (5–10 min), indicating micelle formation. Spectra were run after a 10 min temperature equilibration to 25°C in the cell compartment. At least three recordings were made for each sample.

Estimates of the fractions of α -helix, β -sheet and random coil were determined by using analytical methods developed by Yang's group (Chen *et al.*, 1972, 1974; Wu & Yang, 1976). Reference values for the ellipticity of helix, β - and unordered structures were taken from the computed data of Chen *et al.* (1974) by using five reference proteins. Eqn. (15) of Chan *et al.* (1974) was solved for six parameters, the fraction of helix (f_H), the fraction of β structure (f_β), the average peptide length of helical segments ($\bar{n}$) and the half band widths of the three ellipticity extrema of the circular-dichroism spectrum of α -helix at 223, 207 and 194 nm. Experimental ellip-

ticities at 1.25 nm intervals between 240 and 201.25 nm (i.e. 32 data points) were used and the coefficients were computed by the non-linear least-squares method by using the NLWOOD non-linear least-squares curve-fitting program from McMaster University CDC VIM Library and the CDC 6400 computer at the McMaster University computing centre.

Assays. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Enzyme activity was measured as described previously (Webster *et al.*, 1979). To determine the quantity of phospholipid present in the vesicle portions (diffusion experiments) the entrapment procedure was followed with control samples in which phosphate buffer was replaced with borate buffer so that phospholipid phosphate could be determined. There was no detectable loss of phospholipid after passage through the column.

Radioactivity. Samples were added directly to phase-combining-system scintillation 'cocktail' and counted for radioactivity on the ^{32}P channel of a Beckman LS-200B liquid-scintillation counter.

Results

Interactions of proteins with monolayers of 1:1 phosphatidylserine/phosphatidylcholine at air/water and oil/water interfaces are shown in Figs. 1 and 2 respectively. All proteins were initially at the same molar concentrations in the monolayer subphase and the experimental points show the surface-pressure change induced after the protein injection at various initial monolayer surface pressures. Under the subphase conditions and in the absence of added coenzyme the malate dehydrogenase will be at least 80% dissociated (Wood *et al.*, 1978). The pressure increases resulting from malate dehydrogenase absorption are considerably greater than those caused by the other two proteins at all surface pressures and at both air/water and oil/water interfaces. The intercept of each curve on the abscissa indicates the maximum limiting surface pressure for the absorption of each protein.

Each initial surface pressure corresponds to a certain area per molecule occupied by the phospholipid polar head group in the monolayer. To consider the monolayer as a close representation of half of a bilayer, the area per phospholipid molecule and the surface pressure of the monolayer should be comparable with those in the bilayer. The area per molecule for liquid crystalline phospholipid bilayers of phosphatidylserine or phosphatidylcholine has been estimated to be about 7 nm (Luzzati & Husson, 1962; Reiss-Husson, 1967; Ohki & Ohki, 1976). This corresponds to surface pressures of about 0.2 mN/cm and 0.4 mN/cm at the air/water and oil/water interfaces respectively in Figs. 1 and 2. In this

Fig. 1. Changes in surface pressure induced by protein interaction at the air/water interface of phosphatidylserine/phosphatidylcholine monolayer

Measurements were made as described under 'Methods'. All proteins were injected into the subphase to a final concentration of $0.15\text{ }\mu\text{M}$ and the change in film pressure was recorded until it became constant (usually 30 to 60 min). The subphase was 100 mM-sodium/potassium phosphate buffer, pH 7.0, at 22°C . The data include results combined from five separate experiments. Symbols: O, mitochondrial malate dehydrogenase; ●, mitochondrial malate dehydrogenase plus 1 mM-NADH; Δ, cytochrome c; ▲, ribonuclease A.

context the monolayer at the oil/water interface is a closer approximation to the bilayer condition, since at the oil/water interface the phospholipid acyl chains are fluid in a hydrocarbon phase and the film is more expanded (Ohki *et al.*, 1976; Ohki & Ohki, 1976). The major difference between a monolayer at the oil/water interface and a bilayer is the disruption of van der Waals attractions between acyl chains in the monolayer caused by the insertion of oil into the chain region (Nagle, 1976). However, additional forces will be re-established between the oil hydrocarbon and phospholipid acyl chains, which would pose a comparable barrier to protein penetration.

Under monolayer conditions that most closely approximate to the bilayer, i.e. initial pressures of 0.2 mN/cm at the air/water interface and

0.4 mN/cm at the oil/water interface, there were no pressure changes caused by ribonuclease injection, the cytochrome *c* curves intercepted the axis at these pressures and the malate dehydrogenase curves show considerable pressure increases above these initial values. In particular at the oil/water interface malate dehydrogenase was absorbed to a surface pressure approaching 0.51 mN/cm, which is the collapse pressure of the monolayer. The lower maximum surface pressures caused by malate dehydrogenase when coenzyme was added to the subphase may be caused by inhibition of the enzyme dissociation, which results from coenzyme binding (Shore & Chakrabarti, 1976; Bleile *et al.*, 1977; Webster *et*

Fig. 2. Changes in surface pressure induced by protein interaction at the oil/water interface of a phosphatidylserine/phosphatidylcholine monolayer

The oil was hexadecane and other conditions were as in Fig. 1. The symbols are defined in the legend to Fig. 1.

al., 1979). Addition of NADH alone to the subphase caused a slight increase in surface pressure, suggesting that subsequent absorption may be influenced by initial coenzyme binding to the monolayer.

In Table 1 the $^{22}\text{Na}^+$ self-diffusion rates and $^{22}\text{Na}^+$ permeability coefficients of 1:1 phosphatidylserine/phosphatidylcholine unilamellar vesicles after incubation with the three test proteins are presented. It is expected that any disruption in the continuity of the vesicles hydrophobic regions, which may be caused by the penetration of protein, would decrease the barrier to cation diffusion. The permeability changes agree closely with the monolayer penetration studies: dissociated malate dehydrogenase had the greatest effect, cytochrome *c* caused a slight increase in the diffusion rate when compared to the control and no significant change was observed with ribonuclease. In these experiments the binding of coenzyme to the malate dehydrogenase completely inhibited the enhancement of $^{22}\text{Na}^+$ diffusion that was caused by the dissociated enzyme. Therefore it is possible that the interaction of NADH plus malate dehydrogenase with the monolayer does not signify a penetration by coenzyme-bound enzyme into a bilayer membrane.

Circular dichroism

Our observations suggest that the malate dehydrogenase monomer can penetrate the hydrophobic-chain region of phospholipid membranes; therefore we were interested in determining whether conformational changes accompanied the interaction. It was not possible to use spectrophotometric methods to investigate the enzyme conformation after interaction with vesicles because of the high lipid to enzyme ratio and the consequent suspension turbidity. Dodd (1973) demonstrated by n.m.r. spectroscopy that mitochondrial malate dehydrogenase interacted with the hydrophobic acyl chains of lysophosphatidylcholine. According to Dodd this interaction stabilized the dilute enzyme in a manner similar to our own observation of stabiliza-

Table 1. Diffusion of $^{22}\text{Na}^+$ out of unilamellar phosphatidylserine/phosphatidylcholine vesicles

Experimental conditions are described under 'Methods'. Proteins were added to $1\mu\text{M}$ and NADH to 0.4 mM. Diffusion constants were calculated as described by Papahadjopoulos & Watkins (1967). The total capture of Na^+ by vesicles was $0.104\mu\text{-equiv./}\mu\text{mol}$ of phospholipid. Permeability coefficients were calculated on the basis of an area of $1800\text{cm}^2/\mu\text{mol}$ of phospholipid and a $^{22}\text{Na}^+$ concentration difference of $30\mu\text{-equiv./cm}^3$. Data are the means of three separate experiments carried out in duplicate.

Addition	Protein/ phospholipid molar ratio	Self diffusion (% of total/h per μmol of lipid)	$10^{-12} \times$ Permeability coefficient (s/cm)
None	0.0002	0.189 ± 0.04	1.01
Malate dehydrogenase	0.0002	0.551 ± 0.10	2.94
Malate dehydrogenase + NADH	0.0002	0.187 ± 0.03	1.00
Ribonuclease A	0.0002	0.212 ± 0.05	1.13
Cytochrome <i>c</i>	0.0002	0.285 ± 0.04	1.52

Fig. 3. Circular-dichroism spectra of mitochondrial dehydrogenase

Measurements were made as described under 'Methods' by using a cell with a 0.5 mm path length with the samples in 100 mM-sodium/potassium phosphate buffer, pH 7.0, at 25°C. The experimental spectra used for computation were the average of three typical recordings with appropriate base lines subtracted. This is particularly important when using NADH, as the nucleotide alone is optically active in the 200–250 nm region (Cross & Fisher, 1969). The solid lines show the experimental spectra and the broken lines show the computer-fitted spectra. (a) Mitochondrial malate dehydrogenase at 120 μg/ml; (b) mitochondrial malate dehydrogenase at 130 μg/ml, 0.8% bovine lysophosphatidylcholine; the form of the spectrum with 1.5% lysophosphatidylcholine was very similar; (c) mitochondrial malate dehydrogenase at 130 μg/ml, 0.4 mM-NADH; (d) malate dehydrogenase at 130 μg/ml, 0.4 mM-NADH, 1.5% egg lysophosphatidylcholine.

tion after mixing enzyme subunits with phosphatidylserine/phosphatidylcholine vesicles (Webster *et al.*, 1979). The form of association between enzyme and phospholipid may be similar for lysophosphatidylcholine micelles and phosphatidylserine/phosphatidylcholine vesicles.

In Fig. 3 circular-dichroism spectra of mitochondrial malate dehydrogenase are compared before and after interaction with NADH and lysophosphatidylcholine. After dilution to 120 μg/ml, the enzyme is about 40% dissociated (Wood *et al.*, 1978). Addition of NADH causes full reassociation into dimers. Table 2 shows the computed values (see under 'Methods') for the fractional amounts of α -helix, β -structures and random coil corresponding to each of the spectra in Fig. 3. When the enzyme dimerizes in the presence of NADH or interacts with lysophosphatidylcholine conformational changes resulting in increased α -helix occur. Eberhardt & Wolfe (1975) report no significant change in the 200–250 nm region of the spectrum caused by coenzyme binding in itself. In the presence of both NADH and lysophosphatidylcholine the total change of α -helix was less.

We have been unable to demonstrate unequivocally that lysophosphatidylcholine interacts specifically with enzyme monomers. However, some observations suggest that this may be the case. The previous inactivation studies of Dodd (1973) indicated interaction between lysophosphatidylcholine and mitochondrial malate dehydrogenase subunits. Also, although a conformational change was measured when dimerized enzyme with bound coenzyme interacted with lysophosphatidylcholine (Fig. 3c) the change in α -helix was less than that without NADH addition.

Discussion

We have demonstrated previously (Webster *et al.*, 1979) that mitochondrial malate dehydrogenase monomer, but not the dimer, interacts reversibly

Table 2. Computed values of α -helix and β -structure of mitochondrial malate dehydrogenase

Experimental spectra from Fig. 3 were used for the analysis. The analysis assumes that only three structural forms contribute to the circular-dichroism spectra (Chen *et al.*, 1974). Helix and β -structures were calculated from the best fit and the percentage of random coil was calculated after substitution of the values for helix and β -structures according to the equation: $[\theta] = f_H X_H + f_\beta X_\beta + f_R X_R$, where $[\theta]$ is the mean residue ellipticity, f is the fraction of helix (H), β -form (β) or random-coil form (R) and X_H , and X_β and X_R are the reference values for pure helix (dependent on $\bar{n}$), β -form and random-coil form respectively (Chen *et al.*, 1974). Estimation of $\bar{n}$, the average peptide length of helical regions, is shown although these values may be imprecise by this analysis (Chen *et al.*, 1972, 1974).

Sample	Helix (%)	β -structure (%)	Random coil (%)	$\bar{n}$
Enzyme alone	52	15	33	9.7
Enzyme + 0.8% lysophosphatidylcholine	66	3	31	9.1
Enzyme + 1.5% lysophosphatidylcholine	68	2	30	9.9
Enzyme + 0.4 mM-NADH	66	9	25	7.8
Enzyme + 0.4 mM-NADH + 1.5% lysophosphatidylcholine	57	7	36	11.4

with charged phospholipid vesicles in a manner that enhances subunit stability. The present studies reinforce our previous suggestions that the interaction of the enzyme monomer with phospholipid membranes involves hydrophobic penetration and may involve conformational change.

The absorption of mitochondrial malate dehydrogenase into phospholipid monolayers, and the ability of the enzyme to stimulate leakage of $^{22}\text{Na}^+$ out of phospholipid vesicles was compared with the corresponding effects of cytochrome *c* and ribonuclease A on these parameters. It has previously been shown that ribonuclease A associates with phospholipid surfaces in an ionic manner and with no hydrophobic penetration even at very high protein/phospholipid ratios (Kimelberg, 1976). In agreement with these previous observations ribonuclease A caused minimal increases in monolayer surface pressure and was without effect on the permeability of vesicles to $^{22}\text{Na}^+$. The nature of the interaction of cytochrome *c* with phospholipid membranes has been the subject of some controversy (Das & Crane, 1964; Ulmer *et al.*, 1965; Gulik-Krzywicki *et al.*, 1969; Paphadjopoulos & Kimelberg, 1973; Ohki *et al.*, 1976; Ohki & Ohki, 1976). The loose association of cytochrome *c* with the mitochondrial inner membrane and the close dependence of cytochrome *c* binding to model membranes on the prevailing ionic conditions suggested that the interaction was predominantly if not solely of an electrostatic nature. It now seems probable that an initial ionic interaction is followed by a small conformational change and then partial penetration of the oxidized form of cytochrome *c* into the membrane hydrophobic region (Vanderkooi *et al.*, 1973; Rishpon & Miller, 1977). Our data also suggest a 'borderline' interaction between cytochrome *c* and phospholipid membranes with limited hydrophobic penetration.

In contrast with ribonuclease A and cytochrome *c*, mitochondrial malate dehydrogenase absorbed into the phosphatidylserine/phosphatidylcholine monolayers up to high film pressures, approaching the collapse pressure of the film at the oil/water interface. Under conditions in which the malate dehydrogenase was at least 80% dissociated the enzyme stimulated $^{22}\text{Na}^+$ leakage from 1:1 phosphatidylcholine/phosphatidylserine vesicle matrix to considerably higher rates than either of the other two proteins. Both of the above observations confirm our suggestion (Webster *et al.*, 1979) that the mitochondrial malate dehydrogenase monomer interacts with the hydrophobic interior of phospholipid membranes.

A conformational change may be anticipated when a hydrophilic protein is transferred to an apolar environment (Hammes & Schullery, 1970; Segrest, 1977). The changes in secondary structure reported in Fig. 3 and Table 2 can be related to the

different states of association of the enzyme monomer. At the concentration used and with no additions the enzyme will be about 40% dissociated (Wood *et al.*, 1978) and the spectrum corresponding to this state indicates the lowest α -helical content. Either dimerization in the presence of NADH (Bleile *et al.*, 1977; Wood *et al.*, 1978) or interaction with lysophosphatidylcholine results in an increase in the amount of α -helix. The latter effect may be compared with the interactions of other proteins containing amphipathic helices (Andrews *et al.*, 1976; Segrest *et al.*, 1976; Epand *et al.*, 1977).

We have suggested that the transport of mitochondrial malate dehydrogenase from its site of synthesis on cytosolic ribosomes into the mitochondrial matrix involves diffusion of enzyme subunits across the mitochondrial membranes with fixation in the matrix as dimers, which are unable to interact with phospholipid (Webster *et al.*, 1979). The demonstration that malate dehydrogenase monomer can interact with the hydrophobic interior of a phospholipid membrane supports this suggestion. Further the conformational state of the monomer changes on interaction with phospholipid and on dimerization. These changes might occur during transport of the enzyme. Dimerization on the matrix side of the membrane might provide the thermodynamic drive to fix the enzyme in mitochondria and perhaps pull the monomer out of the membrane. The interaction may be compared with a similar situation where antibody has been shown to react with antigen across a bilayer membrane (Bartford *et al.*, 1968). Conditions that favour dimerization and sequestration of enzyme in the matrix may involve internal pH (Mitchell & Moyle, 1969), binding of substrate (Bleile *et al.*, 1977; Wood *et al.*, 1978) or binding to other enzyme complexes (Halper & Srere, 1977; Fahien *et al.*, 1979).

The model requires some monomer in the matrix. This could be provided by a small amount of monomer existing in equilibrium with dimer; the amount could be less than that present in the cytosol, meaning that inward diffusion would be down a concentration gradient. Since mitochondria multiply by growth and division, there will always be a pool of matrix enzymes. Even in the pro-mitochondria of anaerobic yeast (Criddle & Schatz, 1969), a basal level of matrix enzymes is maintained (Satrustegui & Machado, 1977). Although the initial interaction of enzyme with mitochondria might involve a precursor, a sequence at the subunit-subunit-binding site may confer specificity towards the mitochondrial membrane.

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