Selective permeability of rat liver mitochondria to purified malate dehydrogenase isoenzymes *in vitro*

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1. The mitochondrial malate dehydrogenase from rat liver has been purified to a state of homogeneity as judged by starch-gel electrophoresis and the cytoplasmic isoenzyme has been obtained in a partially purified state. 2. Inhibition of the isoenzymes by sulphite has been studied. 3. In mitochondria loaded with sulphite, the catalytic activity of the (partially inhibited) internal malate dehydrogenase has been measured by addition of oxaloacetate to the suspension medium and observation of the consequent decrease in fluorescence of NADH. 4. Addition of mitochondrial malate dehydrogenase to suspensions of mitochondria loaded with sulphite resulted in an increase in the level of intramitochondrial enzymic activity as measured by the above technique. Addition of the cytoplasmic isoenzyme did not result in such an increase. 5. These results show that mitochondria in suspension are permeable to the mitochondrial malate dehydrogenase but not to the cytoplasmic isoenzyme. 6. This conclusion has been confirmed by direct measurement of a decrease of enzyme activity in solution and an increase inside the mitochondria after incubation of organelles in solutions containing mitochondrial malate dehydrogenase. No such effect was observed with the cytoplasmic isoenzyme. 7. Some features of the permeation process have been studied.

Most of the protein components of mitochondria are coded by the nuclear genome and synthesized on cytoplasmic ribosomes (Schatz & Mason, 1974). There is, therefore, increasing interest in the processes by which cytoplasmic translation products are incorporated into mitochondria and in the factors that dictate the final subcellular location of particular protein species. We have previously studied this problem in relation to the isoenzymes of aspartate aminotransferase (Marra *et al.*, 1977a,b, 1978) and have shown that, *in vitro*, mitochondria are permeable to the mitochondrial isoenzyme but not to the cytoplasmic form. These results suggest that it is the native form of mitochondrial aspartate aminotransferase that is transported, by a presently unknown mechanism, into mitochondria and that the unique subcellular location of the isoenzyme (Baumber & Doonan, 1976) arises from selectivity of the permeation process.

It seemed to be of importance to extend these studies to other proteins to assess the generality of the previous observations; malate dehydrogenase has been chosen for this purpose. Christie & Judah (1953) originally showed that malate dehydro-

genase is present both in the mitochondria and the cytosolic fraction of rat liver. The two enzymes were subsequently found to differ in catalytic (Delbrück *et al.*, 1959) and electrophoretic (Wieland *et al.*, 1959; Sophianopoulos & Vestling, 1960) properties and in their behaviour on ion-exchange chromatography (Thorne, 1960). It is now clear that animal tissues in general contain an acidic malate dehydrogenase in the cytosol and a basic isoenzyme in the mitochondria; moreover, these proteins represent distinct gene products (for a review, see Banaszak & Bradshaw, 1975). It has also been shown (van Heyningen *et al.*, 1974) that mitochondrial malate dehydrogenase is coded by the nuclear genome and therefore synthesized on cytoplasmic ribosomes (Bingham & Campbell, 1972). Hence, as for aspartate aminotransferase (Marra *et al.*, 1977a,b, 1978), the situation exists where a pair of isoenzymes are both synthesized in the cytoplasm but one member of the pair is subsequently transferred specifically to the mitochondria. We show in the present paper that isolated mitochondria are permeable to mitochondrial malate dehydrogenase but not to the cytoplasmic

isoenzyme. This, in conjunction with the previous observations with aspartate aminotransferase, may suggest that selective mitochondrial permeability is at least one of the factors involved in selection of proteins for incorporation into the organelles *in vivo*. A preliminary account of some of this work has been published (Doonan *et al.*, 1979).

Materials and methods

Materials

Oxaloacetic acid was obtained from Sigma. The solid was stored at -20°C and solutions, which were always freshly prepared, were kept in ice; under these conditions, no decarboxylation to pyruvate could be detected in the time required to complete a set of experiments. Ion-exchange celluloses were from Whatman Biochemicals, Maidstone, Kent, U.K. and Sephadexes from Pharmacia Fine Chemicals, Uppsala, Sweden. Rotenone was from F. P. Penick and Co., New York, NY, U.S.A. All other materials were from Sigma. Male white Wistar rats weighing approx. 250 g were used throughout.

Mitochondria

Rat liver mitochondria were isolated as previously described (Klingenberg & Slenczka, 1959) and mitochondrial protein was determined by the method of Waddell (1956).

Measurement of malate dehydrogenase activity

The substrate solution (2 ml) consisted of 0.1 M-triethanolamine/HCl buffer, 2 mM-oxaloacetate and 0.2 mM-NADH at pH 7.5. The solution was incubated at 25°C in a cuvette of 1 cm path length contained in the cell holder of a Unicam SP.30 u.v. spectrophotometer. Reaction was started by addition of enzyme and the decrease in absorbance at 340 nm was recorded as a function of time. One unit of enzyme activity was defined as the amount catalysing the reduction of 1 μmol of oxaloacetate/min.

Isolation of malate dehydrogenase isoenzymes

The methods used were based on those previously employed for isolation of the isoenzymes of aspartate aminotransferase from pig heart (Banks *et al.*, 1968; Barra *et al.*, 1976).

1. Extraction of the isoenzymes from 1 kg of rat liver was as described by Barra *et al.* (1976) except that streptomycin sulphate (1% w/v) was added to the extract to aid in clarification.

2. Separation of cytoplasmic from mitochondrial isoenzymes (both aspartate aminotransferases and malate dehydrogenases) was effected by ion-exchange chromatography on CM-cellulose CM-23.

3. Further purification of mitochondrial malate dehydrogenase was by sequential ion-exchange chromatography on CM-Sephadex C-50 and QAE-Sephadex A-50 as described by Barra *et al.* (1976)

except that the latter column was equilibrated with 10 mM-Tris/HCl buffer, pH 8.7; the enzyme passed through the column unretarded. The enzyme from this step showed one major contaminant on starch-gel electrophoresis at pH 6.2. Final purification was achieved by affinity chromatography on Blue Sepharose CL-6B (Thompson *et al.*, 1975). The column (0.9 cm $\times$ 15 cm) was equilibrated with 20 mM-Tris/HCl buffer, pH 7.0, containing 0.4 mM-EDTA and 20 μM -2-mercaptoethanol. Elution of enzyme was attempted with the same buffer containing 3 mM-NADH, by using a linear gradient generated from 20 ml of starting buffer + 3 mM-NADH and 20 ml of 50 mM-2-amino-2-methylpropane-1,3-diol-HCl buffer, pH 9.0, + 3 mM-NADH, and finally by using a linear gradient generated from 20 ml of 2-amino-2-methylpropane-1,3-diol/HCl buffer and 20 ml of the same buffer containing 1 M-KCl. The enzyme was eluted by the second gradient at a KCl concentration of about 0.3 M.

4. Cytoplasmic malate dehydrogenase was isolated from the unabsorbed fraction from step 2 by precipitation with acetone, fractionation with $(\text{NH}_4)_2\text{SO}_4$, and ion-exchange chromatography on CM-cellulose CM-52 at pH 5.1 as described by Banks *et al.* (1968).

Loading of mitochondria with sulphite

The method was based on procedures previously reported (Crompton *et al.*, 1974; Passarella *et al.*, 1977a). Mitochondria were suspended in 10 ml of a medium containing 0.25 M-sucrose, 20 mM Tris/HCl, 1 mM-EGTA, 2.8 mM-sodium sulphite and 20 μg of rotenone at pH 7.25. After 2 min at room temperature, the suspension was diluted to 50 ml with ice-cold medium with sulphite omitted. Mitochondria were collected by centrifugation and the resulting pellet was washed with sulphite-free medium. A second sample of mitochondria was subjected to the same set of treatments but with sulphite-free medium throughout.

Measurement of intramitochondrial malate dehydrogenase activity

Mitochondria (about 2 mg of protein) were incubated in 1 ml of a medium containing 0.25 M sucrose, 20 mM-Tris/HCl, 1 mM-EGTA and 2 μg of rotenone at pH 7.25 in the cell of an Eppendorf photometer model 1101 M and the fluorescence due to NADH was recorded; filters were used to produce excitation in the range 313–360 nm and to measure emission in the range 430–3000 nm. Oxaloacetate was added to the appropriate concentration and the initial rate of decrease of fluorescence was measured. Variations in conditions for particular experiments are indicated in the legends to the Figures.

Results

Purification of malate dehydrogenase isoenzymes

The mitochondrial isoenzyme purified finally by chromatography on Blue Sepharose CL-6B gave rise to a single cathodal band on starch-gel electrophoresis at pH 6.2 (Barra *et al.*, 1976) whether stained for protein or for enzymic activity. The specific activity was about 950 units $\cdot$ mg⁻¹; contamination with aspartate aminotransferase activity was less than 0.01%. Purification of the cytoplasmic isoenzyme was less satisfactory. Large losses of activity occurred during an abortive attempt to use heat treatment as a purification method; subsequently a substantial proportion (60%) of the activity co-precipitated with aspartate aminotransferase on (NH₄)₂SO₄ fractionation and was rejected. Hence, although the product obtained after the final ion-exchange chromatography was not pure, insufficient material was available to allow further purification. On starch-gel electrophoresis at pH 8.0 two major protein bands of roughly equal intensity were observed migrating towards the anode but only the more rapidly migrating band stained for enzymic activity. However, no contamination with mitochondrial malate dehydrogenase could be

detected after staining heavily overloaded starch gels for enzymic activity; contamination with aspartate aminotransferase activity was less than 0.1% as judged by direct assay. The specific activity of the cytoplasmic malate dehydrogenase preparation was about 450 units $\cdot$ mg⁻¹.

Initial attempts to measure internal malate dehydrogenase levels in intact mitochondria

In previous studies (Marra *et al.*, 1977a) the levels of intramitochondrial aspartate aminotransferase were measured in intact organelles by a method that involved transport of externally added substrates to the site of action of the enzyme. Production of oxaloacetate by the intramitochondrial enzyme was measured by recording the rate of decrease of fluorescence of endogenous NADH consequent on reduction of the oxaloacetate. Attempts were made to employ a similar technique in the present case to measure intramitochondrial levels of malate dehydrogenase. Mitochondria were incubated in an appropriate medium and then oxaloacetate was added. Oxidation of NADH occurred, as shown in Fig. 1, but the rate of decrease of fluorescence was strongly dependent on the external concentration of

Fig. 1. Failure to measure internal malate dehydrogenase activity in suspensions of uninhibited mitochondria. Mitochondria (1.43 mg of protein) were incubated in 1 ml of a medium containing 0.25 M-sucrose, 20 mM-Tris/HCl, pH 7.25, 1 mM-EGTA and 2 μ g of rotenone. In (a) and (c) oxaloacetate was added at the points marked OAA and the decrease in fluorescence of NADH was recorded. In (b) and (d), 11.1 μ g of mitochondrial malate dehydrogenase (mMDH) was added 1 min before addition of oxaloacetate. In each case, a tangent was drawn to the curve at time zero and the rates (marked against each tangent) are expressed as the slopes of these tangents in arbitrary units of scale divisions $\cdot$ min⁻¹.

oxaloacetate. No effect on the rate was observed when purified mitochondrial malate dehydrogenase was added externally. These results suggest that, under the conditions used, the rate of entry of oxaloacetate rather than the rate of its enzyme-catalysed reduction was limiting. This is perhaps not surprising given the high activity of malate dehydrogenase and the relatively low rate of transport of oxaloacetate into mitochondria (Gimpel *et al.*, 1973; Passarella *et al.*, 1977a). It was clear that, to demonstrate possible uptake of externally added malate dehydrogenase into mitochondria, it would be necessary to inhibit at least partially the endogenous enzyme. Use of 2-hydroxy dicarboxylic acids for this purpose (Davies & Kun, 1957) was not considered to be possible because of the likely inhibition by these substances of subsequent transport of oxaloacetate. More promising was the use of sulphite, which strongly inhibits the enzyme (Pfleiderer *et al.*, 1956), and which readily enters mitochondria via the dicarboxylic acid carrier (Crompton *et al.*, 1975). No inhibition of transport of oxaloacetate was to be expected since in rat liver mitochondria this substance is transported entirely by the 2-oxoglutaric acid carrier (Passarella *et al.*, 1977b).

Inhibition of malate dehydrogenase isoenzymes by sulphite

As a preliminary to the experiments reported in the following sections, the inhibition of mitochondrial malate dehydrogenase by sulphite was studied in some detail. From the results reported in Fig. 2(a) the K_m value for oxaloacetate was calculated to be $48 \mu\text{M}$, and from those in Fig. 2(b) the K_i value for sulphite was found to be $175 \mu\text{M}$. The

form of the two plots is indicative of a purely non-competitive inhibition of the enzyme by sulphite. At higher concentrations of sulphite (above $300 \mu\text{M}$) the plot of $1/v$ against inhibitor concentration became non-linear.

Detailed kinetic analysis of inhibition of the cytoplasmic isoenzyme was not carried out. It was observed, however, that when sulphite was added to solutions of the two isoenzymes containing equal amounts of enzymic activity, inhibition of the cytoplasmic isoenzyme was slightly greater than that of the mitochondrial form. Sulphite is also, therefore, an effective inhibitor of the cytoplasmic isoenzyme.

Uptake of mitochondrial malate dehydrogenase into mitochondria loaded with sulphite

Figs. 3(a) and 3(b) compare the rate of decrease of fluorescence on addition of oxaloacetate to control and sulphite-loaded mitochondria. It is clear that the observed rate is much less with loaded organelles and presumably represents the residual malate dehydrogenase activity. It should be noted that the degree of inhibition of endogenous enzyme was much greater than the apparent 57% when Fig. 3(b) is compared with Fig. 3(a) since in Fig. 3(a) uptake of oxaloacetate would have been, at least partially, rate limiting (see above). In Fig. 3(c), mitochondrial malate dehydrogenase was added to the suspension before oxaloacetate and resulted in a 40% increase in rate of change of fluorescence; this is interpreted as showing an uptake of enzyme into the mitochondria. In a control experiment (Fig. 3d) with the cytoplasmic isoenzyme, no such increase in rate of change of fluorescence was observed. Repeat measurements of rates of change of fluorescence

Fig. 2. *Inhibition of mitochondrial malate dehydrogenase by sulphite*

The substrate solution (2ml) contained 0.2M-potassium phosphate buffer, pH 7.5 and 0.2mM NADH; the temperature was 25°C . Rates of reaction are expressed in terms of change in absorbance at $340 \text{ nm} \cdot \text{min}^{-1}$. In (a), the concentrations of sulphite were 0 ($\blacktriangle$), 20 ($\circ$), 40 ($\blacksquare$) and 70 ($\square$) μM respectively. In (b), the concentrations of oxaloacetate were 240 ($\triangle$), 60 ($\bullet$) and 30 ($\blacksquare$) μM respectively. In all cases the amount of enzyme added was $1 \mu\text{g}$.

Fig. 3. Measurement of malate dehydrogenase activity in sulphite-loaded mitochondria and the effect of added malate dehydrogenase isoenzymes

In (b)–(d), sulphite-loaded mitochondria (1.87 mg of protein) were incubated as described in the legend to Fig. 1 and the reaction was started by addition of oxaloacetate (OAA; 2 mM) at the points shown. In (c) 11.1 µg of mitochondrial malate dehydrogenase (mMDH) was added and in (d) approximately the same number of units of the cytoplasmic isoenzyme (cMDH) was added 1 min before the substrate. (a) is a control with mitochondria (1.43 mg of protein) not loaded with sulphite. In all cases, the tangents to the initial parts of the curves are shown and velocities (marked against each tangent) are reported as a percentage of that found for uninhibited mitochondria, with values normalized to the common basis of per 1 mg of mitochondrial protein.

under a particular set of conditions were accurately reproducible. In a typical set of four independent measurements the mean rate was 1.80 min^{-1} with a standard deviation of 0.04 min^{-1} . On the other hand, samples of mitochondria prepared on different days gave different rates of change of fluorescence in the absence of external enzyme and showed variations of enhancement of the rate (20–60%) on addition of 11.1 µg of mitochondrial isoenzyme. This presumably reflects minor variations in the procedure for loading the mitochondria with sulphite.

Direct demonstration of selective uptake of isoenzymes into mitochondria

The experiments quoted in the previous section provide a convenient but somewhat indirect method of studying uptake of mitochondrial malate dehydro-

genase into mitochondria. A more direct approach would be to measure the decrease in content of enzyme in supernatants after removal of organelles by centrifugation; this approach was used successfully for aspartate aminotransferase (Marra *et al.*, 1978). A typical set of results obtained in experiments of this type is shown in Table 1. For the cytoplasmic isoenzyme, all the activity added was recovered in the supernatant after sedimentation of the mitochondria; moreover, after treatment with Triton X-100, the activity of the mitochondrial suspension was the same as that of the control without added enzyme. The behaviour in the case of the mitochondrial isoenzyme was different. Here, a small (approx. 4%) but accurately reproducible amount of the enzyme added to mitochondrial suspension was removed from solution. The enzyme

Table 1. *Direct demonstration of uptake of mitochondrial malate dehydrogenase into mitochondria*

The buffer used contained 250 mM-sucrose, 20 mM-Tris/HCl, pH 7.25, and 1 mM-EGTA. The concentration of the mitochondrial preparation (not sulphite-loaded) was $26.4 \text{ mg of protein} \cdot \text{ml}^{-1}$ and that of the mitochondrial malate dehydrogenase was $0.36 \text{ mg} \cdot \text{ml}^{-1}$. Incubation of mitochondria with isoenzyme was for 1 min at 25°C . Mitochondria were collected by centrifugation and the pellets were resuspended in standard buffer with or without 1% Triton X-100 as indicated. Abbreviations: cMDH, cytoplasmic malate dehydrogenase; mMDH, mitochondrial malate dehydrogenase.

Sample	Composition/ml				Total enzyme activity/arbitrary units		
	Buffer	cMDH	mMDH	Mitochondria	Supernatant	Pellet	
						-Triton X-100	+Triton X-100
Control with cMDH	0.90	0.10	—	—	480	—	—
Control with mMDH	0.95	—	0.05	—	678	—	—
Control with mitochondria	0.90	—	—	0.10	0	0	606
Test with cMDH	0.80	0.10	—	0.10	490	0	600
Test with mMDH	0.85	—	0.05	0.10	650	0	681

was not bound to the outer surface of the organelles (assay of the pellet in the absence of Triton X-100) but a clear increase in the amount of internal enzyme was observed (assay of the pellet in the presence of Triton X-100). These results give a direct demonstration of selective uptake of the mitochondrial isoenzyme in native (not sulphite-loaded) mitochondria.

A point worth noting about these experiments, but not at the moment understood, is that the increase in enzymic activity measurable in lysed mitochondria after incubation with mitochondrial malate dehydrogenase is always greater than the amount lost from the supernatant. This suggests the presence of some component in the mitochondria that effectively activates the mitochondrial isoenzyme. Although this observation has not been pursued in detail, preliminary studies have shown that mixing lysed mitochondria with the purified mitochondrial isoenzyme results in a higher total malate dehydrogenase activity than that calculated from the sum of the activities of the samples assayed individually. Dodd (1973) has reported activation of mitochondrial malate dehydrogenase by lipids, particularly lysophosphatidylcholine, and it may be that the gain of activity reported above was due to combination of the enzyme with mitochondrial lipids.

Dependence of the rate of change of fluorescence on amount of externally added enzyme

The experiments reported above, directly demonstrating uptake of the mitochondrial isoenzyme into mitochondria, provided confirmation that the increased rate of change of fluorescence observed after incubation of sulphite-loaded organelles with the mitochondrial isoenzyme was indeed a measure

Fig. 4. *Variation of rate of change of fluorescence with the amount of externally added mitochondrial isoenzyme* Mitochondria loaded with sulphite (1.87 mg of protein) were incubated as described in the legend to Fig. 1. Various amounts of mitochondrial malate dehydrogenase were added 1 min before addition of oxaloacetate and in each case the initial rate of change of fluorescence was determined.

of import of the protein. The fluorescence method was then used to examine certain characteristics of the uptake. In particular the dependence of rate of change of fluorescence on the amount of mitochondrial malate dehydrogenase is shown in Fig. 4. The rate reached a maximum at about $10 \mu\text{g}$ of added enzyme. Controls without added enzyme were carried out before and after every experiment represented in Fig. 4 and were strictly constant, thus showing that no deterioration had occurred in the

integrity of the mitochondria during the course of the set of experiments. As a second type of control, rates of change of fluorescence were determined with externally added cytoplasmic isoenzyme; no effect on the rate was observed even at high levels of external isoenzyme.

On the assumption that the imported enzyme was inhibited to the same extent as the endogenous malate dehydrogenase, then the results in Fig. 4 indicate a maximum increase by 20% in the content of enzyme within the organelles. For reasons to be discussed later this is probably an overestimate.

Dependence of uptake of enzyme on time of preincubation

A set of experiments was carried out under the usual conditions, except that the time of preincubation of the sulphite-loaded mitochondria with external enzyme before addition of oxaloacetate was varied. With times of preincubation of 0, 1 and 2 min, the observed rates of change of fluorescence were 1.90, 1.97, 1.89 min⁻¹ respectively. The rate in the control (without added enzyme) was 1.25 min⁻¹. Hence it appears that uptake was complete in the time required to add the substrate and start making measurements of the fluorescence.

Discussion

It has previously been shown (Marra *et al.*, 1977a,b, 1978) that when isolated mitochondria are incubated with either mitochondrial or cytoplasmic aspartate aminotransferases, the mitochondrial isoenzyme is transported into the mitochondria whereas the cytoplasmic form is excluded; uptake was demonstrated both by measuring increased intramitochondrial enzymic activity when organelles were incubated with native isoenzyme (Marra *et al.* 1977a) and by incorporation of radioactivity when labelled isoenzyme was used (Marra *et al.*, 1978). These observations suggest that the specific sub-cellular location of aspartate aminotransferase isoenzymes is due to selective permeability of the mitochondrial membrane or membranes. To test the generality of this hypothesis we have now extended the studies to malate dehydrogenase isoenzymes. The choice of this enzyme was dictated partly by the existence of distinct cytoplasmic and mitochondrial forms and partly by the likelihood of being able to devise an assay method for intramitochondrial enzyme activity similar to that used previously (Marra *et al.*, 1977a).

Initial attempts to measure the intramitochondrial enzyme activity by addition of oxaloacetate and observation of NADH oxidation were unsuccessful because of rate-limiting transport of the substrate into the mitochondrial matrix (Fig. 1).

Consistently, externally added mitochondrial malate dehydrogenase did not affect the rate of oxidation of NADH. This problem was overcome by the use of mitochondria in which the endogenous malate dehydrogenase activity was partially inhibited by sulphite. Given the relatively low K_i value for inhibition of mitochondrial malate dehydrogenase by sulphite (175 μ M), it is perhaps surprising that the endogenous enzyme was not completely inhibited in sulphite-loaded mitochondria. However, the internal concentration of sulphite in loaded mitochondria is not known with certainty but is probably low (M. Crompton, personal communication). It is also possible that part of the sulphite is bound to other mitochondrial components and is not available for interaction with the malate dehydrogenase; the situation is also complicated by the observed oxidation of intramitochondrial sulphite to sulphate (Crompton *et al.*, 1974). However, assuming that the rate of decrease of fluorescence of NADH on addition of oxaloacetate to sulphite-loaded mitochondria is a measure of residual malate dehydrogenase activity, then the essential observation is contained in Fig. 3, namely that externally added mitochondrial malate dehydrogenase increases this rate whereas the cytoplasmic isoenzyme does not. We interpret these observations to mean that externally added mitochondrial malate dehydrogenase is able to pass into mitochondria in suspension whereas the cytoplasmic isoenzyme is excluded.

The necessity to use sulphite-loaded mitochondria for these experiments raises certain questions about the validity of this interpretation. For example, it is known that NAD⁺ forms an addition complex with sulphite (Pfleiderer *et al.*, 1956; Parker *et al.*, 1978). However, this complex, if formed under the conditions of the present experiment, did not fluoresce. Moreover, when mitochondria were treated with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone under conditions resulting in oxidation of all intramitochondrial NADH, subsequent addition of sulphite did not produce a change in fluorescence (E. Marra and S. Passarella, unpublished work). Hence no contributions to the observed changes of fluorescence were derived from this source. It might also be argued that the increased rate of change of fluorescence in the presence of added enzyme was due to binding of sulphite by external enzyme and consequent relief of internal inhibition. Direct assay of supernatants after removal of organelles by centrifugation failed to detect any inhibition of added cytoplasmic enzyme and only a small loss of added mitochondrial isoenzyme; the latter could be accounted for by import into the organelles. This observation also allows rejection of another possibility, namely that the effect observed with the

mitochondrial isoenzyme was due to oxidation of NADH that had leaked out of the mitochondria. Given that the cytoplasmic isoenzyme remains active in the suspension medium (see above) then oxidation of leaked NADH would have been observed in this case as well had leakage occurred. Finally, the possibility that the observed effect of added mitochondrial isoenzyme was due to an increased rate of translocation of oxaloacetate is ruled out by the results in Fig. 1.

The arguments listed above seemed to support the view that the increased rate of change of fluorescence on adding mitochondrial malate dehydrogenase to mitochondrial suspensions was due to protein import. However, it was considered to be worthwhile to confirm this conclusion by a more direct method. The results of experiments providing this confirmation are given in Table 1. It was shown by direct enzymic assay that, after incubation of mitochondria with mitochondrial malate dehydrogenase, a small (approx. 4%) but accurately reproducible amount of added enzyme was lost from the suspension medium and was compensated for by an increase in the intramitochondrial level of enzyme measurable after lysis with Triton X-100. With the cytoplasmic isoenzyme, on the other hand, no activity was lost from the suspension medium and no increase was observed with the lysed mitochondria. This approach, which had proved successful in previous work (Marra *et al.*, 1978), seemed to provide the supporting evidence required to validate the technically simpler method based on fluorescence measurements. The latter method was therefore used to investigate the dependence of enzyme incorporation on amount added externally and of the rate of the permeation process. The results reported in Fig. 4 show a hyperbolic dependence of the amount of enzyme incorporated on the amount added externally and suggest that the content of enzyme can be increased to about 120% of the level originally present in the organelles. This may be an overestimate since, for reasons discussed above, the degree of inhibition of imported enzyme is likely to be less than that of the endogenous enzyme; it was not possible to measure the degree of inhibition by lysis of the mitochondria and direct assay since the dilution on lysis resulted in reversal of inhibition. It might be argued that the limit reached in the rate of change of fluorescence in the experiments summarized in Fig. 4 was due to limiting entry of oxaloacetate rather than to saturation of enzyme content in the organelles. This was not the case since the absolute rate measured with sulphite-loaded mitochondria in the presence of excess external enzyme was less than that with uninhibited mitochondria in the absence or presence of external enzyme; hence, in the experiment of Fig. 4, the rate of entry of oxaloacetate must have been sufficiently

high to support the rate of the enzyme-catalysed reaction.

Attempts were made to measure the rate of entry of the mitochondrial isoenzyme into mitochondria, but the process was complete in the time taken to mix solutions and start to make observations; the upper limit to the time required for these operations would be about 30s. In this characteristic of rapid entry, as in the other features discussed above, the permeation of mitochondrial malate dehydrogenase shows great similarities to that of mitochondrial aspartate aminotransferase (Marra *et al.*, 1977a,b, 1978). Given that a convenient quantitative method for measuring uptake has been devised in the latter case, with radioactively labelled enzyme (Marra *et al.* 1978), it will be more fruitful to study the detailed mechanism of permeation through the mitochondrial membrane system with aspartate aminotransferase rather than malate dehydrogenase. The essential point that emerges from this and the previous work is that, in two cases, and hence probably more generally, native mitochondrial matrix proteins are able to pass into intact organelles *in vitro* whereas the corresponding cytoplasmic isozymes are excluded.

The use of the word 'native' in this context requires qualification. The possibility should certainly not be excluded that, for example, it is the monomer (native malate dehydrogenase is dimeric) that is able to pass through the mitochondrial membrane, or that a conformational change occurs during or before the translocation process. These possibilities have been incorporated into a model for selective location of malate dehydrogenase isoenzymes proposed by Webster *et al.* (1979). These authors claim that the porcine mitochondrial isoenzyme dissociates into monomers at low concentration and in the monomeric form interacts with phospholipids with some structural reorganization; the cytoplasmic isoenzyme does not dissociate under the same conditions. Translocation of the monomer is therefore envisaged with subsequent dimerization within the mitochondrion, as a result of higher enzyme concentration and of the presence of nicotinamide nucleotides, preventing exit. It is significant that, on the basis of the dissociation constant given by Webster *et al.* (1979), the mitochondrial enzyme would have been entirely in the monomeric form at the concentrations used in the experiments reported here. The situation is complicated, however, by a conflicting report (Jaenicke *et al.*, 1979) that the mitochondrial malate dehydrogenase does not dissociate even at very low protein concentrations. It is to be hoped that the disagreement between these reports will be resolved.

It is interesting to consider our conclusions about the transport of 'native' proteins into mitochondria in the broader context of the movement of proteins

through membranes from their point of synthesis to their site of action. In the case of proteins secreted from cells, the signal hypothesis has provided a plausible mechanism for some aspects of the transport process (Blobel & Dobberstein, 1975; Campbell & Blobel, 1976). It should be noted, however, that it is central to the idea of the signal hypothesis that the *N*-terminal portion of the growing polypeptide chain passes through the membrane concomitantly with the synthesis of the remaining part of the molecule by membrane bound ribosomes; that is, transport and protein synthesis are necessarily simultaneous events. There is growing evidence that transport of proteins into organelles does not follow this pattern. For example, it has been clearly shown that in the case of chloroplasts, although the small subunit of ribulose biphosphate carboxylase is synthesized as a precursor, transport into the organelles occurs after the completion of polypeptide synthesis (Highfield & Ellis, 1978). An envelope carrier hypothesis was put forward to account for these observations, according to which a class of proteins exists in the chloroplast envelope that recognizes proteins synthesized in the cytoplasm but destined for translocation into the chloroplast. Similarly, studies of the biosynthesis of glutamate dehydrogenase in rat liver (Kawajiri *et al.*, 1977; Godinot & Lardy 1973) and of general matrix proteins in *Neurospora crassa* (Harmey *et al.*, 1977) have led to the view that completed translation products are transported from the cytoplasm into the mitochondria. In two of these reports (Kawajiri *et al.*, 1977; Harmey *et al.*, 1977) it was suggested that minor, but unspecified, post-translational modifications occur during transport of the proteins from a cytoplasmic pool into the mitochondria.

More recent work has confirmed the idea that translation products are released into a cytoplasmic pool before being translocated into the mitochondria but it is now becoming clear that many of these proteins are synthesized as larger precursors. This has been shown for the intermembranal enzyme cytochrome *c* peroxidase (Maccacchini *et al.*, 1979a) for subunit V of the integral membrane component cytochrome *bc₁* (Côté *et al.*, 1979) and for the three largest of the cytoplasmically synthesized polypeptides of the oligomycin-sensitive ATPase (Maccacchini *et al.*, 1979b). As far as matrix enzymes are concerned, synthesis as a precursor has been shown for citrate synthase (Harmey & Neupert, 1979) and for carbamoyl-phosphate synthetase (Raymond & Shore, 1979; Mori *et al.*, 1979); Mori *et al.* (1979) demonstrated processing of the precursor by a mitochondrial membrane preparation. Finally, tentative evidence has been produced for a precursor form of mitochondrial aspartate aminotransferase (Sonderegger *et al.*, 1979). On the other hand, neither the integral protein

of the ADP/ATP translocator system (Zimmermann *et al.*, 1979a) nor apocytochrome *c* (Zimmermann *et al.*, 1979b) is synthesized as a precursor of higher molecular weight. Hence it seems that no single mechanism is likely to exist for the direction of cytoplasmically-synthesized components to mitochondria and for their incorporation into the appropriate site within the organelle.

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