Kinetic studies of the uptake of aspartate aminotransferase and malate dehydrogenase into mitochondria *in vitro*

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1. Kinetic measurements of the uptake of native mitochondrial aspartate aminotransferase and malate dehydrogenase into mitochondria *in vitro* were carried out. The uptake of both the enzymes is essentially complete in 1 min and shows saturation characteristics. 2. The rate of uptake of aspartate aminotransferase into mitochondria is decreased by malate dehydrogenase, and vice versa. The inhibition is exerted by isoenzyme remaining outside the mitochondria rather than by isoenzyme that has been imported. 3. The thiol compound β -mercaptoethanol decreases the rate of uptake of the tested enzymes; inhibition is a result of interaction of β -mercaptoethanol with the mitochondria and not with the enzymes themselves. 4. The rate of uptake of aspartate aminotransferase is inhibited non-competitively by malate dehydrogenase, but competitively by β -mercaptoethanol. 5. The rate of uptake of malate dehydrogenase is inhibited non-competitively by aspartate aminotransferase and by β -mercaptoethanol. 6. β -Mercaptoethanol prevents the inhibition of the rate of uptake of malate dehydrogenase by aspartate aminotransferase. 7. These results are interpreted in terms of a model system in which the two isoenzymes have separate but interacting binding sites within a receptor in the mitochondrial membrane system.

It is well known that the majority of mitochondrial proteins are synthesized in the cell cytosol and then imported into the organelles as completed translation products (Doonan *et al.*, 1984). In many cases the proteins are synthesized as precursors of higher M_r than the mature form, and import into mitochondria is accompanied by proteolytic processing; indeed in recent years there has been some success both in reproducing these processes *in vitro*, i.e. by synthesizing precursors in cell-free systems programmed with mRNA and observing uptake and processing of these precursors by added mitochondria, and in measuring protein import *in vivo* (for a review see Doonan *et al.*, 1984). It is not easy, however, to manipulate these systems in such a way to gain insights into the molecular mechanism of uptake of proteins into mitochondria. Our approach has been different. We have shown that two mature mitochondrial isoenzymes, namely aspartate aminotransferase (Marra *et al.*, 1977, 1978)

and malate dehydrogenase (Passarella *et al.*, 1980), are selectively internalized into mitochondria, whereas the cytosolic forms are not taken up. The relative simplicity of the systems involved has allowed investigation of some of the characteristics of the uptake process; for example, uptake of the proteins has been shown to depend on the transmembrane pH gradient (Passarella *et al.*, 1982, 1983).

One of the outstanding problems in this field is the possible existence and nature of receptor sites in the mitochondrial membrane system involved in protein import. Initial evidence for the existence of such binding sites on the mitochondrial outer membrane has been given for the ADP/ATP carrier (Zimmerman & Neupert, 1980) and for apocytochrome *c* (Hennig & Neupert, 1981); Miura *et al.* (1982) have used a different approach. They showed that the pre-piece of the precursor of ornithine carbamoyltransferase from rat liver is

more basic than is the mature protein. The existence of receptor sites was then proposed in the light of inhibition caused by basic protein such as protamines and histones, but not by acidic proteins, of uptake of the precursor protein.

On the basis of results such as those quoted above, it has become accepted that receptors exist for protein import into mitochondria (see, e.g., Schatz & Butow, 1983), but the evidence is by no means extensive, and very little work has been done on the nature of these putative receptors. In particular, the more valuable criteria used to demonstrate the existence of transport systems as well as of receptors, that is the existence of saturation kinetics and the sensitivity of the uptake to externally added compounds, have not so far been produced.

In the present paper the kinetics of the uptake of both aspartate aminotransferase and malate dehydrogenase are reported, as are the effects of compounds of different types, such as competitor isoenzyme and thiol compounds, on the rate of enzyme uptake. The results reported are consistent with the existence of a common receptor region for both aspartate aminotransferase and malate dehydrogenase. Moreover, inhibition studies support the existence of two different binding sites within the receptor region for the two enzymes as well as of the presence of mercaptoethanol-sensitive groups in the mitochondrial membrane system involved in the import mechanism.

Materials and methods

Materials

Mitochondrial aspartate aminotransferase (EC 2.6.1.1) (M_r 92000) and malate dehydrogenase (EC 1.1.1.37) (M_r 66000) were purified from rat liver as previously described [Porter *et al.* (1981) and Passarella *et al.* (1980) respectively] and were pure as judged by starch-gel electrophoresis at pH 6.2. Rat liver mitochondria were prepared and when necessary loaded with sulphite as described by Passarella *et al.* (1980).

For treatment of mitochondria with β -mercaptoethanol, the organelles after the second centrifugation were suspended in the normal medium (250 mM-sucrose, 20 mM-Tris/HCl, 1 mM-EDTA, pH 7.25) at a concentration of 2 mg of protein/ml and β -mercaptoethanol was added to a final concentration of 20–50 nmol/mg of mitochondrial protein. The suspension was shaken for 2 min at 20°C, after which the mitochondria were sedimented by centrifugation at 10000g for 10 min and then resuspended in the normal medium (see above). When loading of mitochondria with sulphite was carried out, β -mercaptoethanol was added 2 min after sulphite.

Mitochondrial protein was determined as described by Waddell (1956).

All the reagents used were of the highest purity available.

Measurements of the rate of uptake of isoenzymes into mitochondria

This was done in both cases by methods in which the intramitochondrial enzyme activity is first measured and then the increase in this activity is determined after addition of the purified isoenzymes to the organelles. For malate dehydrogenase it is necessary to use mitochondria loaded with sulphite, in which the endogenous enzyme is partially inhibited (Passarella *et al.*, 1980). The details of the assay methods and the conditions under which they provide a true measure of intramitochondrial enzyme activity have been given by Marra *et al.* (1977) for aspartate aminotransferase and by Passarella *et al.* (1980) for malate dehydrogenase.

It was not possible previously to measure the rate of uptake of the enzyme owing to the difficulty of measuring the initial rate of decrease of fluorescence. Thus modification was made to the experimental apparatus to measure kinetics of uptake as well as to improve confidence and reproducibility of each assay. A Perkin-Elmer LS 5 spectrofluorimeter was used equipped with an accessory (5212 4999) which allows direct addition of the substrates or enzymes by injection without opening the cover. Rapid mixing was obtained by using a micro-stirrer (Laurence-RKD-Scientific Ltd., London, Ont., Canada) which gives mixing times as short as 0.5–1 s.

Experimental traces were obtained with maximum reproducibility, this reproducibility decreasing with ageing of mitochondrial preparations. When necessary, mathematical computer treatment of the curves was used to compare the rate in the course of intramitochondrial NAD(P)H oxidation. The duration of each set of experiments ranged was 1–2 h, and controls during the course of experiment were done to test mitochondrial integrity. Mitochondrial preparations, mainly after loading with sulphite, can differ with respect to the absolute rate of change of fluorescence; however, the variability does not affect the results reported here, since each set of experiments was done with a single preparation of mitochondria.

The independence of the initial rate of NAD(P)H oxidation of the rate of oxoglutarate uptake was once again verified (Marra *et al.*, 1977) by testing the increase of the rate of decrease of fluorescence caused by NH_4Cl addition and activity of intramitochondrial glutamate dehydrogenase. This showed a saturating intramitochondrial oxoglutarate concentration under the experimental conditions tested.

Any differences in the conditions used for assay compared with those previously published are given in legends to the Figures.

Results

In Fig. 1 uptake of aspartate aminotransferase into fresh mitochondria (a) and of malate dehydrogenase into mitochondria loaded with sulphite (b) is shown in terms of the percentage stimulation of rate of change of fluorescence compared with the control (in the absence of external enzyme) as a function of time of preincubation of the mitochondria with isoenzyme before addition of the substrate which starts the reaction. The percentage stimulation of rate of change of fluorescence is a direct measure of amount of isoenzyme taken up. As previously reported (Marra *et al.*, 1977; Pasarella *et al.*, 1980) uptake of both enzymes was essentially complete after 1 min, but at shorter times linear (up to 20 s) or hyperbolic increase in uptake with time was observed. When the same results were re-plotted in terms of $2.3 \times \log[S_\infty/(S_\infty - S_t)]$

against time, where S_∞ and S_t are the percentage stimulation at completion of the uptake and at time t respectively, the plot was linear up to a time of 40 s, i.e. the reaction followed first-order kinetics. The first-order rate constant was 2.6 min^{-1} and the corresponding half-life was 0.27 min for aspartate aminotransferase, and 3.7 min^{-1} and 0.19 min for malate dehydrogenase.

The dependence of the rate of uptake of both aspartate aminotransferase and malate dehydrogenase on the amount of externally added enzyme was investigated by using double-reciprocal plots (Fig. 2). Saturation kinetics were found; K_m values (i.e. the amount of the enzyme that gives half-maximum rate of uptake) were 11 nM and 65 nM respectively for aspartate aminotransferase (Fig. 2a) and malate dehydrogenase (Fig. 2b). It should be noted that no comparison is possible between the K_m and $V_{\max}$ values, given that fresh or sulphite-loaded mitochondria were used in the two experiments, depending on which isoenzyme was involved. However, the occurrence of saturation kinetics is in favour of the existence of a receptor in

Fig. 1. Time course of uptake of aspartate aminotransferase and malate dehydrogenase into mitochondria (a) Mitochondria (1.1 mg of protein) were incubated in 2 ml of a standard medium consisting of 250 mM-sucrose, 1 mM-EDTA, 20 mM-Tris/HCl, pH 7.25, containing $2 \mu\text{g}$ of rotenone and 1 mM-sodium arsenite, at 20°C . Aspartate was added to a final concentration of 12.5 mM. After 1 min of incubation, aspartate aminotransferase (3.4 mM) was added and preincubated for the times shown before starting the assay for intramitochondrial aspartate aminotransferase activity by the rapid addition of 2-oxoglutarate (3 mM). Results are expressed as the percentage increase in rate of change of fluorescence, measured by the slopes of tangents drawn to the initial parts of the experimental curves as compared with that of the control reaction without added isoenzyme. A separate control was performed for every time of incubation. In the inset the experimental data are reported as logarithmic plot of aspartate aminotransferase uptake by rat liver mitochondria (see the text). (b) Mitochondria loaded with sulphite (2 mg of protein) were incubated as in (a), except that sodium arsenite was not present. Malate dehydrogenase (15.15 nM) was added, and after the time intervals shown oxaloacetate (2 mM) was added to start the assay of intramitochondrial malate dehydrogenase activity. Results are expressed as in (a). In the inset the experimental data are reported as logarithmic plot of malate dehydrogenase uptake by rat liver mitochondria (see the text).

Fig. 2. Dependence of the rate of uptake of aspartate aminotransferase (a) and malate dehydrogenase (b) into mitochondria on increasing enzyme concentrations

The rate of uptake of aspartate aminotransferase (a) and malate dehydrogenase (b) were measured as reported in Fig. 1: 20s incubation of enzymes with fresh mitochondria (1.1 mg of protein; a) or sulphite-loaded mitochondria (2.2 mg of protein; b) was allowed. The rate of uptake (v) is expressed as % stimulation/min.

the mitochondrial membrane system involved in the transport process. Thus, in order to gain further insight into this putative receptor, possible inhibition of the rate of uptake of both the purified isoenzymes was investigated. Use was made of mitochondrial aspartate aminotransferase or malate dehydrogenase purified from rat liver, and of thiol compounds such as β -mercaptoethanol, dithiothreitol, thiomalate and cysteine.

Typical results establishing the occurrence of inhibition of the rate of uptake of aspartate aminotransferase by mitochondrial malate dehydrogenase or by β -mercaptoethanol as well as of the rate of uptake of malate dehydrogenase by mitochondrial aspartate aminotransferase or by the thiol compound are reported in Table 1.

Some explanation is necessary of the experimental approach used. For aspartate aminotransferase (Expt. 1), fresh mitochondria were incubated in the cell of a spectrofluorimeter set to record the fluorescence of intramitochondrial NAD(P)H. At the times indicated, aspartate and oxoglutarate were added, uptake of which into the organelles provided the substrates for aspartate aminotransferase; enzyme activity was then monitored by the decrease in fluorescence of NAD(P)H, as previously described (Marra *et al.*, 1977) (line 1). Line 2 shows the effect of adding aspartate aminotransferase to the suspension 20s before oxoglutarate. The increase in the rate of change of fluorescence, 33% in this experiment, compared with line 1 is due to uptake of the enzyme into the mitochondria. On the other hand, when aspartate aminotransferase and the tested compounds were added together to the mitochondrial suspension (lines 4 and 7), the stimulation of the rate of change of fluorescence decreased to 17 and 16% for malate dehydrogenase and β -mercaptoethanol respectively. No inhibition was found when the tested compounds were added after the enzyme (lines 5 and 8). Given that added compounds alone did not

affect the control (cf. line 1 with lines 3 and 6), this means that their presence had inhibited the uptake of the aspartate aminotransferase.

A similar set of experiments using malate dehydrogenase is reported in Expt. 2. In this case it was necessary to use mitochondria loaded with sulphite to inhibit endogenous malate dehydrogenase activity partially, otherwise uptake of oxaloacetate into the organelles becomes rate-limiting (Pasarella *et al.*, 1980). Aspartate aminotransferase was used as a possible inhibitor together with β -mercaptoethanol. All the compounds tested decreased the rate of uptake of malate dehydrogenase if added together with the enzyme (lines 4 and 7), but had no significant effect if added after the enzyme (lines 5 and 8). Hence it may be concluded that aspartate aminotransferase and β -mercaptoethanol effectively inhibit uptake of malate dehydrogenase into mitochondria.

In both cases inhibition by dithiothreitol was found, whereas cysteine and thiomalate gave no significant effect on the rate of uptake of either enzyme.

It is also noteworthy that, for aspartate aminotransferase, an alternative method for measurement of uptake into mitochondria has been developed by using enzyme radiolabelled by reduction of the apoprotein-cofactor linkage with NaB^3H_4 (Marra *et al.*, 1978). This technique has also been used to confirm the inhibitory effect of the tested compounds, but further use of this method was limited by the very low specific radioactivity of the labelled protein, which makes it necessary to use very high amounts of enzyme (about 10 times the reported K_m). As far as the uptake of malate dehydrogenase is concerned, no labelled enzyme that can be imported into mitochondria has been available to date.

Although the most obvious interpretation of the results reported is in terms of a direct inhibition by one isoenzyme of uptake of the other, two other ex-

Table 1. *Effect of different compounds on the rate of uptake of aspartate aminotransferase and malate dehydrogenase into mitochondria*

Expt. 1: inhibition by malate dehydrogenase and β -mercaptoethanol of the rate of uptake of aspartate aminotransferase into mitochondria. Mitochondria (Mit) (1.2mg of protein) were incubated as described in Fig. 1(a). After 1 min of incubation, aspartate (Asp) was added to a final concentration of 12.5mM. Other additions were made at the times indicated. The final concentrations of mitochondrial aspartate aminotransferase (mAAT), malate dehydrogenase (mMDH), β -mercaptoethanol (ME) and 2-oxoglutarate (OG) were 5.43nM, 5.68nM, 12.5 μ M and 3mM respectively. The decrease in fluorescence after the rapid addition of OG was continuously recorded in each test, and the initial rate of the reaction was measured as reported in Fig. 1(a). Values are expressed as percentages of the rate of the control reaction without added enzyme, to which the value of 100 arbitrary units/scale divisions was given. Expt. 2: inhibition by aspartate aminotransferase and β -mercaptoethanol on the rate of uptake of malate dehydrogenase into mitochondria. Sulphite-loaded mitochondria (Mit) (1.2mg of protein) were incubated as described in Fig. 1(b). After 1 min of incubation, additions were made at the times reported. The final concentrations of mAAT and mMDH added in this case were 6.25nM and 11.36nM respectively. ME and OAA were added to final concentrations of 12.5 μ M and 2mM respectively. The fluorescence decrease after the rapid addition of OAA was continuously recorded, and the initial rate of the reaction was measured as described in Fig. 1(b). Values are reported as in Expt. 1.

Expt. 1						Expt. 2			
Line	Additions				Relative rate of change of fluorescence	Additions			Relative rate of change of fluorescence
	<i>t</i> = 0	+60s	+20s			<i>t</i> = 0	+20s		
1	Mit	Asp	—	OG	100	Mit	—	OAA	100
2	Mit	Asp	mAAT	OG	133	Mit	mMDH	OAA	139
3	Mit	Asp	mMDH	OG	100	Mit	mAAT	OAA	100
4	Mit	Asp	mAAT	OG	117	Mit	mMDH	OAA	117
5	Mit	Asp	+ mMDH mAAT	OG	133	Mit	mMDH	OAA	139
				+ mMDH				+ mAAT	
6	Mit	Asp	ME	OG	100	Mit	ME	OAA	100
7	Mit	Asp	mAAT	OG	116	Mit	mMDH	OAA	122
8	Mit	Asp	+ ME mAAT	OG	133	Mit	mMDH	OAA	139
				+ ME				+ ME	

planations were considered. Firstly, the presence in the system of the competitor isoenzyme may have interfered with uptake of substrates into the organelles, thus making substrate entry at least partially rate-limiting. Secondly, the presence of the competitor isoenzyme may have partially collapsed the pH gradient, which is known to be essential for protein uptake (Passarella *et al.*, 1982, 1983). That neither of these was the case was shown by experiments carried out in parallel with those reported in Table 1 (but not given in detail), where greater concentrations of the isoenzymes whose uptake was being measured were used, but in the presence of a constant concentration of inhibitor. In both cases a progressive increase in stimulation of rate of change of fluorescence was observed with increased concentration of test isoenzyme, which would not have been the case if either of the above processes had been responsible

for the effect reported in Table 1. (Note that, in the presence of rotenone, intramitochondrial flavo-protein-linked substrates are the energy-supplying metabolites supporting the uptake process.)

For clarity, the inhibition studies are separately examined below.

Mutual inhibition of uptake of aspartate aminotransferase and malate dehydrogenase in vitro

Features of the inhibition process. Uptake of isoenzymes into mitochondria is very rapid, as shown in Fig. 1; hence it was possible that the inhibition observed in the experiments described in Table 1 was due to some effect exerted by inhibitor that had already entered the mitochondria rather than to an effect on the putative mitochondrial receptor. To clarify this issue, two sets of experiments were carried out (results not shown) in which the effect of time of preincubation of mitochondria with in-

hibitor before addition of the test isoenzymes was investigated.

Under the conditions used, the uptake of the inhibitor isoenzyme would have ranged from zero at simultaneous addition to approaching saturation at 60s preincubation before addition of the test enzyme. Nevertheless, the subsequent uptake of the test isoenzyme was affected equally in all cases, showing that it was the inhibitor remaining outside the mitochondria rather than that which had been accumulated that was effective in inhibiting uptake of the test isoenzyme; that is, the inhibitors exert their effects at the level of the membrane receptors rather than on the capacity of the mitochondria for total protein uptake.

Nature of the inhibition process. To obtain information about the mechanism of the mutual inhibition, the time course of the uptake of both enzymes was measured in the absence or presence of a fixed concentration of the inhibitor enzyme. Inhibition decreasing with time was found in both cases, i.e. the rate of uptake was inhibited, rather than the capacity of the mitochondria for protein uptake, in agreement with the above-mentioned results.

In Fig. 3 the results are given of experiments where the rate of uptake of aspartate aminotransferase was measured as a function of increasing enzyme concentration in the absence or presence of different concentrations of malate dehydrogenase. For each determination the rate of change of fluorescence was measured and compared with that of a control in the absence of added aspartate aminotransferase. Results are expressed as percentage stimulation/min of the rate of change of fluorescence and were obtained by measuring the rate of decrease of fluorescence in 20s in either the

absence or the presence of inhibitor. Fig. 3(a) shows a double-reciprocal plot of $1/(\% \text{ stimulation/min})$ against $1/[\text{aspartate aminotransferase}]$ in the absence or the presence of malate dehydrogenase. Maximum stimulation was decreased in the presence of malate dehydrogenase, whereas the K_m value was constant. Thus the results obtained are consistent with a non-competitive inhibition by malate dehydrogenase of the rate of uptake of aspartate aminotransferase.

Further confirmation that the inhibition was non-competitive is given in Fig. 3(b), where a Dixon plot of $1/(\% \text{ stimulation/min})$ against concentration of malate dehydrogenase is shown. K_m and K_i values for aspartate aminotransferase and malate dehydrogenase were found to be about 12 and 6nM

An analogous set of experiments in which the inhibitory effect of aspartate aminotransferase on the rate of uptake of malate dehydrogenase was determined is shown in Fig. 4. The kinetic behaviour was again indicative of non-competitive inhibition by the criteria used, as judged by both the double-reciprocal plot (Fig. 4a) and the Dixon plot (Fig. 4b). The K_m and K_i values for malate dehydrogenase and aspartate aminotransferase were 64.7 and 4.3nM respectively in this experiment.

Inhibition of the rate of uptake of aspartate aminotransferase and malate dehydrogenase by β -mercaptoethanol

Features of the inhibition process. When use is made of chemical compounds in mitochondrial systems, it is important to investigate carefully the mechanism by which the tested compound exerts its influence. That β -mercaptoethanol inhibits the

Fig. 3. Kinetic analysis of the inhibition by malate dehydrogenase of the rate of uptake of aspartate aminotransferase. Rates of change of fluorescence (v) were measured in 20s as described in Fig. 1(a) by using 2.2mg of mitochondrial protein. Results are reported as % stimulation/min. (a) Double-reciprocal plots of results obtained with aspartate aminotransferase in the absence ($\bigcirc$) or presence of ($\triangle$) 1.9nM-, ($\blacksquare$) 3.8nM-, ($\bullet$) 5.8nM- and ($\blacktriangle$) 7.7nM-malate dehydrogenase. (b) Dixon plots of the same results obtained with ($\blacktriangle$) 3.4nM-, ($\bullet$) 5.6nM- and ($\blacksquare$) 10.3nM-aspartate aminotransferase.

Fig. 4. Kinetic analysis of the inhibition by aspartate aminotransferase of the rate of uptake of malate dehydrogenase. Rates of changes of fluorescence (v) were measured in 20 s as described in Fig. 1(b), with sulphite-loaded mitochondria (1.2 mg of protein). Results are expressed as % stimulation/min. (a) Double-reciprocal plot obtained with malate dehydrogenase in the absence ($\circ$) or presence of ($\blacksquare$) 2.7 nM-, ($\blacktriangle$) 6.1 nM- or ($\bullet$) 9.1 nM-aspartate aminotransferase. (b) Dixon plot of the same results obtained with ($\bullet$) 7.1 nM-, ($\blacktriangle$) 10.6 nM-, ($\blacksquare$) 17.8 nM and ($\circ$) 35.6 nM-malate dehydrogenase.

rate of enzyme uptake owing to the presence of a thiol group in the molecule is demonstrated by prevention experiments, not reported in this paper, which showed that little or no change occurs in the rate of uptake of the tested enzymes when β -mercaptoethanol was added to mitochondria in the presence of *N*-ethylmaleimide or mersalyl, under conditions in which these thiol reagents do not inhibit the enzyme uptake (see Marra *et al.*, 1979a). With β -mercaptoethanol, preliminary investigations were also made to establish that the inhibition was due to a direct interaction of this compound with the mitochondrial membrane and not with the enzyme molecule. Thus, if aspartate aminotransferase and malate dehydrogenase were incubated with mercaptoethanol (at an inhibiting concentration) before addition to the mitochondrial suspension, no change in the increase in rate of change of fluorescence was observed owing to the addition of enzymes. The results quoted above suggest that the effect of β -mercaptoethanol is exerted on the mitochondria rather than on the isoenzymes. That this is so was established by experiments not reported here in detail, in which mitochondria were treated with β -mercaptoethanol as described in the Materials and methods section and then their ability to internalize the isoenzymes was examined. Mitochondria pretreated with β -mercaptoethanol failed to take up aspartate aminotransferase; it was possible to reverse the inhibitory effect of β -mercaptoethanol by adding the penetrant thiol reagent *N*-ethylmaleimide or the non-penetrant mercurial mersalyl (Fonjo, 1978), which themselves do not change the rate of decrease of fluorescence in the absence of any

added enzyme; in fact the addition of aspartate aminotransferase after these compounds resulted in a stimulation of the rate of change of fluorescence. Analogous experiments have been done for malate dehydrogenase, and essentially the same results were obtained.

Nature of the inhibition by β -mercaptoethanol of the rate of uptake of aspartate aminotransferase and malate dehydrogenase. The nature of the inhibition by β -mercaptoethanol on the uptake of aspartate aminotransferase and malate dehydrogenase was investigated by varying the concentration of isoenzyme added in the presence or absence of the inhibitor. A difference in the sensitivity to inhibition by β -mercaptoethanol of uptake of aspartate aminotransferase and malate dehydrogenase was found in control experiments in which very low β -mercaptoethanol concentrations were used. For aspartate aminotransferase, inhibition was found with the lowest concentration of β -mercaptoethanol tested (5 μ M); on the contrary, uptake of malate dehydrogenase was not measurably decreased by 5 μ M- β -mercaptoethanol. Moreover, if 30 s preincubation of mitochondria with 5 μ M- β -mercaptoethanol was allowed, complete inhibition was found of aspartate aminotransferase uptake, which could be reversed by adding mersalyl, but not enzyme, whereas the uptake of malate dehydrogenase was not affected. When higher concentrations of β -mercaptoethanol were used, complete inhibition of malate dehydrogenase uptake was found after 30 s incubation, which was reversed only by mersalyl but not by the dehydrogenase.

Thus inhibiting concentrations of mercapto-

ethanol were used in investigating the uptake of aspartate aminotransferase (Fig. 5) and malate dehydrogenase (Fig. 6). The experimental data were plotted both as double-reciprocal plots and as Dixon plots. It is clear that β -mercaptoethanol inhibits the uptake of aspartate aminotransferase and of malate dehydrogenase in different ways. For the former enzyme, the presence of β -mercaptoethanol does not affect the maximum rate of uptake, as shown by the common ordinate intercepts, but changes the affinity of the enzyme for the putative receptor, as shown by the different intercepts on the abscissa (Fig. 5a), i.e. it increases

the apparent K_m value, in agreement with a competitive inhibition; this is confirmed by the Dixon plot (Fig. 5b), the K_i value for β -mercaptoethanol being about $15 \mu\text{M}$. For malate dehydrogenase the presence of β -mercaptoethanol does not change the apparent K_m value, but decreases the maximum rate of uptake, in agreement with a non-competitive kind of inhibition (Figs. 6a and 6b). The K_i value was about $20 \mu\text{M}$.

Given that uptake of aspartate aminotransferase is more sensitive than uptake of malate dehydrogenase with respect to β -mercaptoethanol, and that no inhibition of uptake of the latter occurs

Fig. 5. Kinetic analysis of the inhibition by β -mercaptoethanol of the rate of uptake of aspartate aminotransferase. Assays were carried out as described in Fig. 2, with 1.2 mg of mitochondrial protein. (a) Double-reciprocal plots of results obtained with aspartate aminotransferase in the absence (○) or presence of (■) $7.5 \mu\text{M}$ -, (▲) $12.5 \mu\text{M}$ - or (●) $25 \mu\text{M}$ - β -mercaptoethanol. (b) Dixon plots of the same results obtained with (▲) 6.3 nM -, (●) 12.3 nM - or (■) 18 nM -aspartate aminotransferase.

Fig. 6. Kinetic analysis of the inhibition by β -mercaptoethanol of the rate of uptake of malate dehydrogenase. The experiments were carried out as described in Fig. 3, except that the amount of mitochondrial protein was 1.2 mg. (a) Double-reciprocal plots of the results obtained with malate dehydrogenase in the absence (○) or presence of (■) $7.5 \mu\text{M}$ -, (▲) $12.5 \mu\text{M}$ - or (●) $25 \mu\text{M}$ - β -mercaptoethanol. (b) Dixon plots of the same results obtained with 7.1 (●), 10.6 (▲), 16.6 (■) and 28.5 nM (○) malate dehydrogenase.

Fig. 7. Prevention by β -mercaptoethanol of the inhibition by aspartate aminotransferase of the uptake of malate dehydrogenase. Sulphite-loaded mitochondria (1 mg of protein) were incubated as reported in Table 1 (expt. 2). After 2 min of incubation, oxaloacetate (OAA; 2 mM) was added to start the reaction. Other additions at times indicated by the arrows were as follows: β -mercaptoethanol (ME), 5 μ M; mitochondrial malate dehydrogenase (mMDH), 9.47 nM; mitochondrial aspartate aminotransferase (mAAT), 4.35 nM. The results are expressed as a percentage increase in rate of change of fluorescence measured by the slopes of tangents drawn to the initial part of the experimental curves, as compared with that of the control reaction without added isoenzyme, to which a value of 100 arbitrary units/scale divisions was given.

up to 5 μ M- β -mercaptoethanol, possible prevention by low concentrations of β -mercaptoethanol of the inhibition shown by aspartate aminotransferase of the rate of uptake of malate dehydrogenase has been tested (Fig. 7). At 5 μ M, β -mercaptoethanol, which completely inhibits the uptake of 4.35 nM-aspartate aminotransferase in the time-scale used (not shown), has no effect on the rate of intramitochondrial NAD(P)H oxidation induced by externally added oxaloacetate, either in the absence (trace b) or in the presence (trace d) of mitochondrial malate dehydrogenase, i.e. it does not inhibit the uptake rate of this enzyme (cf. traces c and d). The inhibition of the uptake of malate dehydrogenase by aspartate aminotransferase (trace f) is completely prevented by β -mercaptoethanol (cf. traces g and h). There was no effect owing to the presence of aspartate aminotransferase and β -mercaptoethanol on the control reaction (see traces a, b, e and h).

Discussion

The objective of the present work was to obtain evidence for the existence of a receptor or receptors involved in the uptake of aspartate aminotransferase and malate dehydrogenase into mitochondria. In view of the possibility to apply to protein import criteria previously used for substrate translocation across the mitochondrial membrane, the kinetics of the uptake of these enzymes have been examined. This was made possible by a modification to the cell compartment of the spectrofluorimeter, allowing direct addition to the cell *in situ*. This technical modification is fundamental to the validity of the present work; thus kinetic measurements were done by using short preincubation times of isoenzyme with mitochondria before addition of substrate.

The rates of uptake obtained may seem unreasonably large, since, in experiments where precursors obtained by protein synthesis in cell-free

systems are processed by mitochondria *in vitro*, half-lives are much longer and incubation periods of 30–60 min are frequently required (see Doonan *et al.*, 1984, and references therein). However, there is evidence that uptake of proteins into mitochondria *in vivo* is very rapid, with half-lives of the order of a few seconds or minutes (see, e.g., Mori *et al.*, 1981; Reid *et al.*, 1982). Hence the rates observed in our model system reflect the rates of the process *in vivo*. The pseudo-first-order constants reported in this paper (measured at 20°C) are of the same order as those determined at 2–9°C for anion uptake into isolated mitochondria (Quagliarriello *et al.*, 1969; Palmieri *et al.*, 1972a,b; Coty & Pedersen, 1975); however, considering a Q_{10} value of 3–4, the rate of uptake of anions can be considered about 10 times greater according to the different size and M_r of the internalized compounds.

The existence of saturation kinetics lends support to the receptor hypothesis. Even if for technical reasons it is not possible to compare the $V_{\max}$ values, which are characteristic of each transport system, just evidence that aspartate aminotransferase and malate dehydrogenase are recognized by the same mitochondrial membrane system is given by their mutual inhibition as well as by inhibition by β -mercaptoethanol. Mutual inhibition of aspartate aminotransferase and malate dehydrogenase has been found to depend on an effect on the rate of uptake, but not on the general capability of mitochondria in taking up proteins.

Mutual inhibition of the uptake suggests a common receptor area for the two enzymes; in view of the non-competitive nature of inhibition, a different binding area for the two enzymes can be proposed in the same membrane region, so that the formation of the complex aminotransferase–receptor–dehydrogenase prevents further internalization. Our results do not, of course, distinguish between the inner or outer membrane as the site for interaction with β -mercaptoethanol as well as of the tested enzyme, but we have previously shown (Marra *et al.*, 1982) that mitoplasts internalize both isoenzymes in a way that is essentially indistinguishable from that found with intact mitochondria, and inhibition of uptake of both aspartate aminotransferase and malate dehydrogenase into mitoplasts has been found; hence an effect of β -mercaptoethanol and competitor enzyme on the inner membrane seems more likely, but cannot be considered definitively established. It may also be the case that there is no clear distinction between the mitochondrial membrane if, as has been suggested (Chua & Schmidt, 1979), protein import occurs at contact sites between the inner and outer membranes.

Different kinds of mitochondria were used in the

two assays; thus the difference found between the K_m for the uptake and the K_i values obtained with the same enzyme, when used as a competitor, cannot be carefully compared. However, a two-step process in enzyme internalization, namely binding of enzyme to the mitochondria (which is responsible for the inhibition found) and subsequent internalization of the bound enzyme into the matrix (see also Marra *et al.*, 1979b, 1980) may be proposed.

For β -mercaptoethanol, our approach to the problem was suggested by the observation that blockage of thiol groups in aspartate aminotransferase (Marra *et al.*, 1979a) or malate dehydrogenase (Passarella *et al.*, 1983) with reagents such as mersalyl effectively prevents internalization of the isoenzymes into mitochondria *in vitro*. One explanation of these observations would be that internalization involves interaction between isoenzyme thiol groups and specific binding regions of receptors in the mitochondrial membrane system. The results reported here lend support to this idea in that β -mercaptoethanol inhibits the internalization process when present in the assay. Removal of the inhibition of β -mercaptoethanol by mersalyl also suggests, in view of the impermeability of the mitochondrial membrane to this compound (Fonjo, 1978), that the proteins bind at the external face of the mitochondrial membrane, where the putative receptor is likely to be located. This experiment also rules out other non-specific effects owing to inhibitor–mitochondrial-membrane interaction, once more verifying the validity of our model system in maintaining controlled experimental conditions (see Doonan *et al.*, 1984). Complete inhibition by β -mercaptoethanol of the uptake of both enzymes was found after 30 s incubation; this inhibition could be reversed by mersalyl, but not by the enzymes tested (namely aspartate aminotransferase). Thus binding of β -mercaptoethanol to mitochondria is tentatively suggested here to produce a time-dependent conformational change in the receptor area in such a way that the enzyme binding sites become inaccessible to the proper isoenzyme. The experiments reported in this paper suggest that different molecules sensitive to β -mercaptoethanol are present in the protein receptor in the mitochondrial membrane, in that both inhibition of the uptake of aspartate aminotransferase can be found without affecting malate dehydrogenase internalization and prevention by β -mercaptoethanol of the inhibition of malate dehydrogenase uptake by aspartate aminotransferase occurs.

It should be noted that mutual inhibition of the uptake suggests a common receptor area for the two enzymes, but could be also due to the possible use of two distinct receptors by aspartate amino-

transferase and malate dehydrogenase, these receptors having the property of binding the proper enzyme as well as the competitor. However, the effect of low concentrations of β -mercaptoethanol in preventing inhibition of uptake of malate dehydrogenase otherwise exerted by aspartate aminotransferase clearly shows that this is not the case.

The difference in the nature of inhibition by β -mercaptoethanol of the rate of uptake of aspartate aminotransferase and malate dehydrogenase strongly confirms that different binding sites are available for the two enzymes.

In conclusion, we favour the occurrence of a common receptor for aspartate aminotransferase and malate dehydrogenase uptake into isolated rat liver mitochondria. In agreement with the different nature of inhibition shown by β -mercaptoethanol on the rate of uptake of aspartate aminotransferase and malate dehydrogenase and with the mutual non-competitive inhibition, a common binding site is suggested to exist for the inhibitor and the transaminase, in accord with competitive inhibition, whereas for malate dehydrogenase the enzyme binding occurs at a site different from that which binds β -mercaptoethanol and aspartate aminotransferase.

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