Uptake of aspartate aminotransferase into mitochondria *in vitro* depends on the transmembrane pH gradient

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1. The effects of various inhibitors of electron transport and of oxidative phosphorylation and the effects of ionophores on the uptake of native aspartate aminotransferase into mitochondria were investigated. 2. Both antimycin and cyanide completely inhibited the uptake of the enzyme. On the other hand, uptake was stimulated by ATP and by oligomycin; however, the stimulation by ATP is inhibited by oligomycin. 3. The effects of ionophores of the valinomycin type in media containing K^+ ions depended on the conditions used. Valinomycin alone stimulated the uptake of the enzyme, but in the presence of phosphate ions uptake was abolished. Nonactin was without effect at a low K^+ concentration, but was stimulatory at 100 mM-KCl. Gramicidin also stimulated the uptake process. 4. Nigericin completely abolished uptake of aspartate aminotransferase into mitochondria. 5. The uptake of the enzyme was decreased by 18% in the absence of inhibitors or ionophores when the external pH was increased from 6.9 to 7.6. 6. These results indicate that ATP is not directly involved in the uptake of aspartate aminotransferase into mitochondria, neither is there a requirement for a cation gradient. Rather the uptake depends on the maintenance of a pH gradient across the mitochondrial inner membrane.

Most of the proteins which constitute a mitochondrion are coded by the nuclear genome, synthesized on cytosolic ribosomes (sometimes but not always as precursors of higher molecular weight than the native forms) and translocated into the organelle after completion of protein synthesis; some aspects of these processes have been reviewed by Neupert & Schatz (1981).

Many details of the mechanism of import of proteins into the mitochondria remain obscure, but attention has recently been focused on the energy-dependence of the process. Nelson & Schatz (1979) have shown that, in yeast cells depleted of mitochondrial ATP, precursors of mitochondrial matrix and inner-membrane proteins accumulate in the cells instead of being processed. It was not shown directly whether the precursors accumulated in the cytosol or the mitochondria, but it has been argued on other grounds (Neupert & Schatz, 1981) that import rather than proteolytic processing is the step requiring ATP. In other experiments (Hallermayer & Neupert, 1976; Zimmermann & Neupert, 1980) it has been shown that the uncoupler carbonyl cyanide

m-chlorophenylhydrazine ('CCCP') prevents import of precursors of mitochondrial proteins into organelles both *in vivo* and when mitochondria are added to cell-free systems programmed to synthesize specific mitochondrial proteins.

Our approach to the study of import of proteins into mitochondria differs from those referred to above. We have shown that mitochondria incubated with purified native mitochondrial aspartate aminotransferase (Marra *et al.*, 1977, 1978) or malate dehydrogenase (Passarella *et al.*, 1980) internalize these proteins, whereas the corresponding cytosolic isoenzymes are not taken up. This selective import of mitochondrial isoenzymes into organelles *in vitro* shows features subsequently established for the process of import of precursors *in vivo*. For example, import of aspartate aminotransferase is completely abolished by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine ('FCCP') (Marra *et al.*, 1980). This model system has therefore been used to probe in more detail the energy-dependence of import of aspartate aminotransferase into mitochondria by examination of the

effects of inhibitors of electron transport and of oxidative phosphorylation and the effect of ionophores. In summary, it is shown that ATP is not directly involved in the process, but that uptake of the protein is dependent on maintenance of a proton gradient across the mitochondrial membrane. A preliminary account of some of the work presented here has been reported previously (Passarella *et al.*, 1981).

Materials and methods

Materials

Rat liver mitochondria were isolated as previously described (Marra *et al.*, 1977) and re-suspended in the appropriate medium (see legends to Figures and Tables). Mitochondrial aspartate aminotransferase was isolated from rat liver as described by Porter *et al.* (1981). Other materials were obtained from Sigma, except for nigericin, which was a gift from Dr. Rita Casadio (Institute of Botany, University of Bologna, Italy).

Assay of aspartate aminotransferase

Assay of enzyme activity in free solution was by the linked method of Karmen (1955), with the substrate concentrations given by Baumber & Doonan (1976). Results were expressed as rates of change of $A_{340} \cdot \text{min}^{-1}$.

Measurement of uptake of the enzyme into mitochondria

This was done by a method which measures the intramitochondrial activity of aspartate aminotransferase and the increase in this activity consequent on the addition of enzyme to the mitochondrial suspension. The details of the method and evidence that it provides a true measure of intramitochondrial aspartate aminotransferase activity have been presented previously (Marra *et al.*, 1977). The only difference in the method used in the present work

was that a Perkin-Elmer spectrophotofluorimeter model 3000 was employed, with excitation and emission wavelengths set at 334 nm and 456 nm respectively; previously a filter fluorimeter had been used.

In all cases except with nigericin, the amount of enzyme added to the mitochondrial suspension was chosen so as not to saturate the uptake; this was done in order to allow detection of small changes in amount of enzyme imported. It should be noted that different samples of mitochondria give rise to different degrees of stimulation of rate of change of fluorescence when enzyme is added externally. Every set of measurements was carried out in a relatively short time; controls were done at the beginning, during and at the end of each experiment, and only differences of not more than 5–10% were accepted. The constancy of the controls guarantees the accuracy of every test.

Results

Effect of inhibitors of oxidation and phosphorylation on the uptake process

It has previously been shown (Marra *et al.*, 1980) that the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone completely inhibits uptake of aspartate aminotransferase into mitochondria. This could be interpreted in terms either of removal of mitochondrial ATP or of dissipation of mitochondrial ion gradients (ΔpH and $\Delta\psi$ in the terminology of Mitchell, 1968). Protons should be the candidate ions for the gradient, even if ΔpH -dependent distribution of several anions across the mitochondrial membrane is mediated by the P_i/OH^- antiport (Palmieri *et al.*, 1970). We have now extended studies to other compounds which affect the process of oxidative phosphorylation.

Results obtained with two inhibitors of electron transport, namely antimycin and cyanide, are given in Tables 1 and 2 respectively. In these, as in all

Table 1. *Effect of antimycin on the uptake of aspartate aminotransferase into mitochondria*

Mitochondria (Mit) (4 mg of protein) were suspended in 2 ml of 0.25 M-sucrose/1 mM-EDTA/20 mM-Tris/HCl (pH 7.25) containing rotenone (2 μg) and 1 mM-sodium arsenite. After 3 min additions were made at the times indicated; the final concentrations of aspartate (Asp) and 2-oxoglutarate (Ox) were 12 mM and 2.8 mM respectively. The quantities of mitochondrial aspartate aminotransferase (mAAT) and antimycin (Ant) added were 5.4 μg and 1 μg respectively. Immediately after addition of 2-oxoglutarate the initial rate of decrease of fluorescence was measured. Values are given as a percentage of the rate of the control with only aspartate and 2-oxoglutarate added.

Additions						Relative rate of change of fluorescence
	$t = 0$	+30 s	+30 s	+1 min	+1 min	
Mit	Asp	—	Ox	—	—	(100)
Mit	Asp	mAAT	Ox	—	—	150
Mit	Asp	—	Ant	—	Ox	100
Mit	Asp	—	Ant	mAAT	Ox	117
Mit	Asp	mAAT	Ant	—	Ox	148

Table 2. *Effect of cyanide on the uptake of aspartate aminotransferase into mitochondria*

The incubation medium was 0.25 M-sucrose/1 mM-Tris/EDTA/5 mM-Tris/HCl (pH 7.25) and the amount of mitochondrial protein was 2 mg. Additions were made to the same final concentrations as in Table 1, except that the amount of enzyme added was 2.7 μ g; the final concentration of KCN was 1 mM. Abbreviations are as in Table 1.

Additions						Relative rate of change of fluorescence
	<i>t</i> = 0	+30 s	+30 s	+1 min	+1 min	
Mit	Asp	—	Ox	—	—	(100)
Mit	Asp	mAAT	Ox	—	—	122
Mit	Asp	—	KCN	—	Ox	72
Mit	Asp	—	KCN	mAAT	Ox	71

experiments reported here, the observed parameter is the rate of decrease of fluorescence of intramitochondrial NADH resulting from addition of aspartate followed by 2-oxoglutarate (the substrate pair for aspartate aminotransferase) to the mitochondrial suspension. The initial rate of change of fluorescence is proportional to the intramitochondrial aspartate aminotransferase activity (Marra *et al.*, 1977); a typical example of the traces obtained is shown in Fig. 1.

Results obtained with antimycin are given in Table 1. The initial rate of change of fluorescence of the mitochondrial suspension when only substrates were added was arbitrarily equated to 100 units $\cdot$ min⁻¹. Addition of mitochondrial aspartate aminotransferase to the suspension after addition of aspartate but before addition of 2-oxoglutarate resulted in an increase of the value by 50%. Addition of antimycin 30 s before the enzyme abolished almost completely the increase in rate of change of fluorescence; that is, it prevented uptake of the enzyme into the organelles. Antimycin added alone did not change the observed rate of change of fluorescence, neither did antimycin when added after addition of aspartate aminotransferase. As a further control, it was found (results not shown) that neither antimycin nor any other of the compounds studied except nigericin had a significant effect on the activity of mitochondrial aspartate aminotransferase measured in free solution. Similar results were obtained with cyanide (Table 2). A stimulation of 22% in the rate of change of fluorescence was obtained on addition of aspartate aminotransferase to the mitochondrial suspension. Addition of cyanide to the suspension 2 min before addition of 2-oxoglutarate caused a decrease in rate of change of fluorescence of 28% compared with the control. The same value was obtained when aspartate aminotransferase was added after addition of cyanide, thus showing that cyanide completely abolished the ability of mitochondria to import externally added enzyme.

The reason for the decreased rate of change of fluorescence in the control with added cyanide

compared with that without cyanide is not clear (similar effects were observed with other compounds; see below), but the conclusion that mitochondria so treated are unable to internalize added aspartate aminotransferase is unambiguous.

As an example of a compound which inhibits ATPase rather than reacting with a component of the electron-transport chain we have used oligomycin. The results are given in Fig. 1 for two different concentrations of inhibitor. At low concentration (Fig. 1b) oligomycin had a small effect on the control reaction, decreasing the rate of change of fluorescence by 20% (cf. traces A and C). On addition of oligomycin followed by aspartate aminotransferase (trace D) the relative rate of change of fluorescence was 133 units $\cdot$ min⁻¹; this represents an increase of 33% over the rate of the control without oligomycin (trace A) and an increase of 66% over the rate of control with oligomycin (trace C); this result suggests that oligomycin stimulates uptake of aspartate aminotransferase into mitochondria. The effect is seen more strongly at higher concentrations of oligomycin (Fig. 1a). The inhibitory effect of oligomycin on the control reaction is greater than at lower concentration (cf. traces A and C), but correspondingly the stimulation on addition of external enzyme is also greater (cf. traces C and D), the rate of change of fluorescence being three times that of the control with oligomycin. The effect of ATP on the uptake of the enzyme and its sensitivity to oligomycin were examined in the experiment reported in Table 3. ATP (1 mM), which itself inhibits the control, strongly increases the uptake of the enzyme from 111 to 148%, the presence of oligomycin strongly decreasing this ATP stimulation. However, oligomycin itself stimulates the uptake of the mitochondrial enzyme, according to Fig. 1.

Effect of ionophores on the uptake process

We have studied the effect on uptake of mitochondrial aspartate aminotransferase into mitochondria of two distinct types of ionophores, namely those of the valinomycin type, which dissipate the

Fig. 1. *Effect of oligomycin on the uptake of aspartate aminotransferase into mitochondria*
The incubation medium and the abbreviations were the same as in Table 1, except for the concentration of Tris/HCl in (b), which was 5 mM. The amounts of mitochondria were 3.3 mg and 3.0 mg in (a) and (b) respectively. Other substances were added at the times indicated and to the same final concentrations as in Table 1, except for aspartate aminotransferase [2.64 μ g in (a) and 2.7 μ g in (b)] and oligomycin [Olig: 5 μ g in (a) and 2 μ g in (b)]. The solid lines are traces of the original records of change in fluorescence as a function of time. Broken lines are tangents drawn to the initial parts of the curves. The slopes of the tangents are expressed as a percentage of that of the control reaction taken as 100. Values in parentheses are the slopes of the tangents expressed as a percentage of that of the control containing oligomycin but not added enzyme.

Table 3. *Effect of ATP on the uptake of aspartate aminotransferase into mitochondria and sensitivity to oligomycin*
The incubation medium and the abbreviations were the same as in Table 1 and the amount of mitochondrial protein was 2 mg. The concentration of ATP was 1 mM; amounts of aspartate aminotransferase and oligomycin (OLIG) were 7.65 and 2 μ g respectively. v , v_{ATP} , $v_{ATP+OLIG}$, v_{OLIG} indicate the rates of decrease of fluorescence in the absence or in the presence of ATP, ATP + oligomycin and oligomycin respectively. Values are given as a percentage of the rate of each control.

Additions						v	v_{ATP}	$v_{ATP+OLIG}$	v_{OLIG}
	$t = 0$	+1 min	+1 min	+30 s	+30 s				
Mit	—	—	Asp	—	Ox	100			
Mit	—	—	Asp	mAAT	Ox	111			
Mit	—	ATP	Asp	—	Ox	59	100		
Mit	—	ATP	Asp	mAAT	Ox	83	148		
Mit	OLIG	ATP	Asp	—	Ox	51		100	
Mit	OLIG	ATP	Asp	mAAT	Ox	71		139	
Mit	—	OLIG	Asp	—	Ox	78			100
Mit	—	OLIG	Asp	mAAT	Ox	106			137

mitochondrial transmembrane potential, and those of the nigericin type, which eliminate the pH gradient but with a corresponding increase in membrane potential. This distinction is based on several types of experimental evidence, but has been particularly clearly shown by Laris *et al.* (1975) and in subsequent work on accumulation of cationic fluorescent probes into mitochondria under different conditions (Johnson *et al.*, 1981).

Results with valinomycin are reported in Fig. 2(a). The presence of valinomycin in the control decreased the observed rate of change of fluorescence by 34% (cf. curves A and C), but the addition of aspartate aminotransferase resulted in an increase in rate of 65% compared with the control without valinomycin (curves A and D) or of 150% compared with control containing valinomycin (curves C and D). Thus treatment of mitochondria with valinomycin appears to stimulate uptake of the enzyme. A different result was obtained if P_i was included (Fig. 2b). P_i alone did not affect the extent of uptake of enzyme into mitochondria (results not shown), but the combination of valinomycin and P_i

inhibited completely the uptake process (cf. traces C and D in Fig. 2b). It has been shown that P_i + valinomycin uncouple mitochondria (Moore & Pressman, 1964). Possible swelling by P_i and valinomycin under the same experimental conditions has been tested, but no significant change in the absorbance occurs, confirming that no damage to the mitochondrial membrane is caused by P_i + valinomycin. The ionophore nonactin has different effects on ion movement into mitochondria, depending on the concentration of K^+ in the suspension medium. For this reason, the effect on uptake of aspartate aminotransferase into mitochondria was studied in media containing 10mM-KCl (Fig. 3a) and 100mM-KCl (Fig. 3b). At low concentration of KCl (Fig. 3a) nonactin decreased the rate of the control (curves A and C), but did not affect the extent of uptake of enzyme into the organelles. Comparison of curves C and D shows a stimulation of the rate of decrease in fluorescence by 59% on addition of aspartate aminotransferase, a value very similar to that (58%) of the stimulation in the absence of nonactin (curves A and B). At high

Fig. 2. Effect of valinomycin on the uptake of aspartate aminotransferase into mitochondria in the absence (a) and presence (b) of phosphate ions

The incubation medium contained 100mM-KCl, 20mM-Tris/HCl (pH 7.0), 1mM-Tris/EDTA, 2 μ g of rotenone and 1mM-sodium arsenite. The amount of mitochondrial protein was 2.6mg. Additions were made at the times shown. The quantities of valinomycin and of enzyme used were 50 μ g and 10.4 μ g respectively. In (b) the final concentration of phosphate ions was 2.5mM. Abbreviations are as in Table 1.

Fig. 3. *Effect of nonactin on the uptake of aspartate aminotransferase into mitochondria*

The incubation medium in (a) contained 10 mM-KCl, 0.23 M-sucrose, 5 mM-Tris/HCl (pH 7.25), 1 mM-Tris/EDTA, 1 mM-sodium arsenite and 2 μ g of rotenone. In (b) sucrose was omitted and the KCl concentration was increased to 100 mM. The mitochondrial protein in both cases was 2.5 mg. Final concentrations of aspartate and 2-oxoglutarate were as in previous experiments. The amount of enzyme added was 2.7 μ g; the amount of nonactin (Non) was 0.28 μ g in (a) and 0.22 μ g in (b). Abbreviations are as in Table 1.

concentration of KCl, on the other hand, nonactin increased the uptake of the enzyme (Fig. 3b). An 88% increase in rate of change of fluorescence was observed when nonactin was added 3 min and enzyme 1 min before the start of the reaction (curves B and C); a greater increase (122%) was observed when nonactin was added 2 min and enzyme 3 min before the start of the reaction. Hence the effect of

nonactin is greater when the time of preincubation with enzyme and ionophore together is increased.

Gramicidin was taken as a final example of this class of ionophore, and the effects of this substance on the uptake of aspartate aminotransferase into mitochondria are shown in Table 4. Gramicidin did not inhibit the control reaction. When enzyme was added to the incubation medium the rate of reaction

Table 4. *Effect of gramicidin on the uptake of aspartate aminotransferase into mitochondria*

The incubation medium was the same as in Fig. 2 and the amount of mitochondrial protein was 5.25 mg. Amounts of aspartate aminotransferase and gramicidin (Gram) added were 6.6 μ g and 0.5 μ g respectively. Abbreviations are as in Table 1.

Additions					Relative rate of change of fluorescence
	<i>t</i> = 1 min	+ 1 min	+ 30 s	+ 30 s	
Mit	—	Asp	—	Ox	(100)
Mit	—	Asp	mAAT	Ox	112
Mit	Gram	Asp	—	Ox	105
Mit	Gram	Asp	mAAT	Ox	162

Fig. 4. *Effect of nigericin on the uptake of aspartate aminotransferase into mitochondria*

The incubation medium contained 100mM-KCl, 20mM-Tris/HCl (pH 7.00), 1mM-Tris/EDTA, 1mM-sodium arsenite and 2 μ g of rotenone. The amount of mitochondrial protein was 3.6mg. Final concentrations of aspartate and 2-oxoglutarate were as in previous experiments. The amount of enzyme used was 20 μ g and the amount of nigericin (Nig) was 0.56 nmol. Abbreviations are as in Table 1.

was increased by 12% in the absence of gramicidin (a low external enzyme concentration was used in this experiment), whereas in the presence of gramicidin an increase of 54% was observed; that is, the presence of gramicidin stimulates uptake of aspartate aminotransferase into mitochondria. As outlined briefly above, nigericin has an action on mitochondrial ion gradients which is distinctly different from that of the other ionophores. This difference is reflected dramatically in its effect on the uptake of aspartate aminotransferase into mitochondria (Fig. 4). Nigericin alone had a stimulatory effect on the control, a reflection of the fact that it has an activatory effect on the enzyme in free solution, but on addition of enzyme externally (curve D) no further stimulation was observed; that is, nigericin completely abolished uptake of the enzyme. On the other hand, if the enzyme was added before

nigericin only a slight diminution of rate was observed (curves C and E). It should be noted that in the particular experiment quoted a large amount of external enzyme was used to maximize the observed effect (i.e. to obtain a saturating uptake of enzyme in the absence of the ionophore). In other experiments at lower external enzyme concentration the same complete abolition of uptake of enzyme by nigericin was observed. *Effect of external pH on the uptake of aspartate aminotransferase into mitochondria* The results reported above indicate that the pH gradient across the mitochondrial membrane is the determining factor in uptake of aspartate aminotransferase (see the Discussion section). Consequently, experiments were carried out on the effect of varying the pH of the suspension medium on the

uptake process. The results are given in Fig. 5. Measurements of the rate of change of fluorescence were made both with and without added aspartate aminotransferase at various pH values in the range 6.9–7.6. The stimulation of the rate consequent on addition of enzyme externally decreased in this pH range by about 18%; that is, more enzyme was incorporated at low than at high pH. One aspect of these experiments is puzzling, namely that the rate of change of fluorescence in the control and in the test samples with added enzyme decreased in the pH range 6.9–7.6 even though the activity of the enzyme measured in free solution did not change significantly. The reason for the apparent decrease in intramitochondrial enzyme activity could be due to a different pH-activity curve of the enzyme in the mitochondrial matrix as a result of the pH difference across the membrane (see Dawson & Selwyn, 1974). It is important to note, however, that at all pH values the magnitude of the increase in the rate of

change of fluorescence on adding enzyme to the incubation medium depended on the amount of enzyme added. Hence, although absolute values of the rates of change of fluorescence at different pH values cannot be compared, differences in percentage increase with respect to controls may be compared with confidence.

Discussion

Previous work has shown that there is an energy-dependence of uptake of proteins into mitochondria, but without considering the problem of the nature of the link between uptake and energy generation (Hallermayer & Neupert, 1976; Nelson & Schatz, 1979; Zimmermann & Neupert, 1980; Marra *et al.*, 1980). Systems *in vivo* for studying protein uptake do not lend themselves very easily to the solution of these problems, since addition of inhibitors of energy generation may have effects on processes other than those of interest.

We have developed a model system in which the transport of mitochondrial aspartate aminotransferase into mitochondria can be observed as an increase in enzyme activity within the organelles (Marra *et al.*, 1977), and we now report the effects on this process of various inhibitors of oxidative phosphorylation and of ionophores.

The inhibitory effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on the uptake of the enzyme has been reported previously (Marra *et al.*, 1980). Further evidence for the energy-dependence of the uptake process is given in Tables 1 and 2, where it is shown that inhibitors of electron transport block the process, completely with cyanide and by about 70% with antimycin; failure of antimycin to abolish the uptake is not surprising, since this substance is known not to inhibit electron transport completely. It should be noted that the fluorimetric assay used in this and in previous work (Marra *et al.*, 1977) requires that the respiratory chain be inhibited with rotenone to prevent oxidation of NADH by electron transport. Hence, given the effect of antimycin and cyanide, it is clear that under the conditions used for the assay substrates oxidizable by FAD-dependent enzymes must produce the energy for the uptake process. Consistently succinate has a stimulatory effect on uptake (results not shown).

Results obtained up to this point did not allow a distinction to be drawn between the direct involvement of ATP or of a transmembrane potential in the uptake process. Evidence that the latter is the determining factor is given in Fig. 2, where it is shown that oligomycin, an inhibitor of ATP synthesis, stimulates the uptake process in a concentration-dependent manner. This suggests that maintenance of a transmembrane proton gradient is

Fig. 5. Effect of external pH on the uptake of aspartate aminotransferase into mitochondria

The buffer used was 5 mM-Hepes [4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid] adjusted to the appropriate pH with Tris. Other components of the incubation medium were 0.24 M-sucrose and 1 mM-Tris/EDTA. The amount of enzyme added in all cases was 15.3 μ g. ●, Rate of change of fluorescence of the control reaction; ▲, rate of change of fluorescence after addition of enzyme to the incubation medium; ■, stimulation of the rate of change of fluorescence.

the essential factor in import of the enzyme into mitochondria. It should be noted that no increase of pH owing to a possible Cl^-/OH^- exchange occurs in the external medium after addition of oligomycin to mitochondria. This conclusion is strongly confirmed by the finding that ATP stimulated the uptake process in an oligomycin-sensitive manner, i.e. ATP appears to stimulate the process by the formation of an ion gradient and not by direct phosphorylation of any component of the mitochondrion or of the enzyme itself. The use of ionophores has allowed confirmation of the conclusion that ion gradients are essential for the process, but more importantly has provided further evidence that it is the proton component of the gradient that is directly involved.

Ionophores of the valinomycin type, in media containing K^+ ions, decrease the $\Delta\psi$ component (in the terminology of Mitchell, 1968), but increase the ΔpH value. With valinomycin alone (Fig. 2a) a considerable increase in uptake of enzyme into mitochondria was observed, whereas in the presence of phosphate ions (Fig. 2b) uptake was abolished. The effects of nonactin on the distribution of K^+ and H^+ depend on concentration of K^+ ions in the incubation medium (Palmieri & Quagliariello, 1969). Consistently, the effect of the ionophore on uptake of aspartate aminotransferase into mitochondria varied with K^+ concentration in the incubation medium (Fig. 3). At a KCl concentration of 10 mM, where the ΔpH value is essentially unaltered (Fig. 3a), uptake of the enzyme is unaffected by the presence of nonactin. However, at higher K^+ concentration (Fig. 3b), where the transmembrane pH gradient is increased, a stimulation of uptake occurred. Finally, gramicidin, which also promotes an increase in ΔpH with a corresponding decrease in $\Delta\psi$ (Chappell & Crofts, 1965), also stimulates uptake of the enzyme into mitochondria. In summary, therefore, ionophores that under the appropriate experimental conditions cause a decrease in $\Delta\psi$ with a corresponding increase in ΔpH stimulate uptake of the enzyme. This suggests that the transmembrane pH gradient provides the driving force for translocation of the protein and underlines the major role played by the inner membrane.

Strong support for this hypothesis was provided by the use of the ionophore nigericin (Fig. 4). Nigericin catalyses an electroneutral exchange of K^+ and H^+ and hence results in collapse of the pH gradient while not affecting $\Delta\psi$. If, as suggested above, the import of aspartate aminotransferase is driven by the pH gradient but is independent of $\Delta\psi$, then nigericin should be inhibitory. That this is the case is shown by the results in Fig. 4. Nigericin added before addition of external aspartate aminotransferase inhibited uptake completely (curves B and D), whereas when the order of additions was

reversed (curve E) the rate was only marginally affected. A final confirmation of the importance of the pH gradient across the mitochondrial membrane was provided by the observation that as the pH of the incubation medium was decreased the amount of enzyme imported into the organelles increased (Fig. 5). This pH gradient could be generated either by electron transport or by hydrolysis of ATP.

The conclusion that the pH gradient across the mitochondrial membrane provides the driving force for uptake of proteins into mitochondria rather than a direct involvement of ATP is not unique in the sense that several other energy-dependent processes, including ion- or substrate-transport processes, have been shown to operate in the same manner (Grinius, 1980; Skulachev, 1980). It is, however, in conflict with the conclusions of Nelson & Schatz (1979). These authors claim that the inability of valinomycin to prevent processing of precursors of mitochondrial proteins in wild-type yeast cells rules out involvement of a membrane potential. It could equally be argued, however, that these results support an involvement of the pH gradient in processing of precursors; it would have been of interest to test the effect of nigericin in this system. A second point raised by Nelson & Schatz (1979), namely that ρ^- mutants, which cannot form an appreciable membrane potential, nevertheless are able to process precursors, is more difficult to reconcile with our conclusions. It may be that these mutants, which have defective inner membranes, behave atypically in respect of processing, or it may be that yeast and animal mitochondria differ in the mechanism of uptake of proteins.

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