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Uptake of aspartate aminotransferase into mitochondria in vitro causes efflux of malate dehydrogenase and vice versa

Salvatore Passarella [1], Ersilia Marra [2], Anna Atlante [2], Maria Barile [1], Shawn Doonan [3] and Ernesto Quagliariello [1]

Addresses: [1] Dipartimento di Biochimica e Biologia Molecolare, Universitá degli Studi Bari, Bari, [2] Centro Studi sui Mitocondri e Metabolismo Energetico, C.N.R., Bari (Italy) and [3] Department of Biochemistry, University College, Cork (Republic of Ireland)

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Incubation of intact mitochondria with aspartate aminotransferase results in efflux of malate dehydrogenase and vice versa. The export process is specific and rapid. It shows saturation kinetics with respect to the effector enzyme consistent with involvement of a receptor for the effector in the mitochondrial membrane system. Export is inhibited by both β-mercaptoethanol and by the metal chelating agent bathophenanthroline; both substances inhibit release of malate dehydrogenase by aspartate aminotransferase competitively whereas for release of aspartate aminotransferase by malate dehydrogenase inhibition is non-competitive. The efflux process is dependent on a trans-membrane pH gradient. Exported enzymes differ from the native forms in their dependence of activity on pH. Export of both aspartate aminotransferase and malate dehydrogenase is effected by incubation of mitochondria with the newly-synthesised precursor of aspartate aminotransferase; this observation provides supporting evidence for the physiological significance of the other results reported here. It is speculated that exported enzymes are on a pathway to degradation, and that coupled uptake and export is involved in the co-ordination of synthesis and breakdown of mitochondrial proteins.

Introduction

The processes by which mitochondrial proteins synthesised in the cell cytosol are translocated into the organelles have been the subject of intensive study in recent years [1–6]. In general, the proteins are synthesised as precursors with N-terminal extensions and these extensions are considered to play a part in both the translocation and the final destination of a particular protein.

Notwithstanding this biosynthetic process, we have shown previously [7–9] that mature mitochondrial aspartate aminotransferase and malate dehydrogenase are internalized when incubated with mitochondria in vitro, and this process mirrors many of the features of the process of precursor uptake, such as dependence on membrane receptors and on the presence of a transmembrane ion gradient. These observations call into question the relevance of the presequence for the transmembrane movement.

Little is known to date about the processes of degradation of mitochondrial proteins and the coordination of synthesis and degradation.

Although mitochondria contain proteolytic enzymes [10,11], it seems that part at least of the degradation process occurs after the proteins have been exported from the mitochondria. For example, it has been shown [12] that lysosomes play a part in proteolysis of mitochondrial proteins, and that exit of proteins from mitochondria is accelerated by import of newly synthesised protein [13].

These observations suggest that specific mechanisms may exist for release of mitochondrial proteins into the cell cytosol as part of the degradation process. Some initial evidence for this has been provided by Miralles and co-workers [14] who showed that incubation of rat liver mitochondria with apocytochrome *c* provoked exit of the holoprotein. We have previously reported [15,16] that incubation of mitochondria with mature aspartate aminotransferase causes exit of malate dehydrogenase activity and vice versa as revealed by detection of enzymatic activity in the extramitochondrial phase. The process is specific in that neither enzyme caused exit of adenylate kinase, glutamate dehydrogenase, isocitrate dehydrogenase, fumarase or ornithine aminotransferase.

Correspondence: S. Passarella, Dipartimento di Biochimica e Biologia Molecolare, via Amendola 165/A, 70126 Bari, Italy.

We report here further characteristics of the process and show that exported enzymes have different kinetic characteristics from the native forms. We also report that the precursor of aspartate aminotrasferase causes efflux of both aspartate aminotransferase and malate dehydrogenase. It is suggested that the exported enzymes may be on a pathway to degradation.

Materials and Methods

Enzymes and mitochondria. Mitochondrial aspartate aminotransferase (EC 2.6.1.1) and malate dehydrogenase (EC 1.1.1.37) were purified from rat liver as previously described [17,9] and were pure as judged by starch gel electrophoresis at pH 6.2 and polyacrylamide gel electrophoresis in the presence of SDS. Rat liver mitochondria were isolated as previously described [18] and mitochondrial protein was measured according to Ref. 19. All the reagents were from Sigma (St. Louis, U.S.A). Solution pH was adjusted to pH 7.2 by using Tris.

Measurement of efflux of mitochondrial enzymes. Measurements were done in both cases by assays linked to oxidation of NADH the process being followed photometrically using a Perkin-Elmer Lambda 5 spectrophotometer with a 3700 Data Station. The software allowed for measurement of the slopes of tangents to the initial part of the progress curves so that rates were calculated as straight lines with regression coefficients of 0.996 or better for ΔA_{340}/min. A stirred cell compartment was used which allowed rapid mixing and addition to the cell without opening the cover.

To measure externalised malate dehydrogenase, NADH (0.2 mM) and oxaloacetate (2 mM) were added to the mitochondrial suspension followed 30 s later by aspartate aminotransferase. Exported malate dehydrogenase catalysed reduction of oxaloacetate by NADH as shown by decrease in absorbance. Exported aspartate aminotransferase was measured by addition of aspartate (12.5 mM), 2-oxoglutarate (3 mM) and NADH (0.2 mM) to the mitochondrial suspension followed 30 s later by malate dehydrogenase. Exported aspartate aminotransferase produced oxaloacetate from the substrate pair and the latter was reduced by NADH catalysed by the added malate dehydrogenase; in this case the enzyme added to the mitochondria is acting not only as an effector of release of aspartate aminotransferase but is also participating in the assay.

The incubation medium in both cases was 0.25 M sucrose, 20 mM Tris-HCl (pH 7.25) and 1 mM Tris-EDTA.

Electrophoresis. Samples for analysis by SDS-polyacrylamide gel electrophoresis [20] were prepared as follows. Mitochondria (about 1.5 mg of protein in each of five Eppendorf tubes) were incubated in a standard medium consisting of 0.25 M sucrose, 20 mM Tris-HCl (pH 7.25), 1 mM EGTA-Tris in a volume of 1 ml.

Effector enzymes were added to the tubes and efflux of intramitochondrial enzymes allowed to proceed for different times. Mitochondria were then sedimented using a refrigerated Haereus Christ microcentrifuge. The supernatants were collected (total of 5 ml), compounds of a molecular weight higher than 100 000 removed using a MX 100 membrane in an Amicon dialysis cell, and then concentrated to 0.5 ml using a PM 10 membrane.

Efflux promoted by the precursor of aspartate aminotransferase. The precursor form of aspartate aminotransferase was synthesised in a reticulocyte lysate system programmed with purified mRNA as previously described [21,22]. The products of cell-free synthesis were incubated with mitochondria in the normal medium for 30 min after which the organelles were removed by centrifugation. The supernatant was tested for activity of aspartate aminotransferase, glutamate dehydrogenase, fumarase and isocitrate dehydrogenase [23–25]. As controls, supernatants were taken from mitochondrial suspensions without added precursor.

Enzyme assays. Aspartate aminotransferase and malate dehydrogenase were assayed as previously described [7,9].

Results

Specific efflux of enzymes from mitochondria

This phenomenon has been demonstrated in two different ways, that is by an electrophoretic method and by activity measurements.

Results obtained by electrophoresis are shown in Fig. 1A. Supernatants from mitochondrial suspensions with no added effector contained no proteins in the 10 000–100 000 molecular weight range (lane e) that is, the mitochondria isolated and incubated as described were not leaky. Lane a contains mature mitochondrial aspartate aminotransferase. Lane b contains the post-mitochondrial supernatant after incubation with aspartate aminotransferase. Residual aspartate aminotransferase is clearly present as is a band of protein with greater mobility at a position corresponding to that of malate dehydrogenase (lane c). Equally, the supernatant from mitochondria incubated with malate dehydrogenase (lane d) contained a protein migrating at the same position as aspartate aminotransferase. It should be noted that the subunit molecular weights of aspartate aminotransferase and malate dehydrogenase are 46 000 and 33 000, respectively.

Confirmation of the identities of exported proteins was obtained by activity measurements as shown in Figs. 1B and 1C. Addition of aspartate aminotransferase to mitochondria caused efflux of an activity,

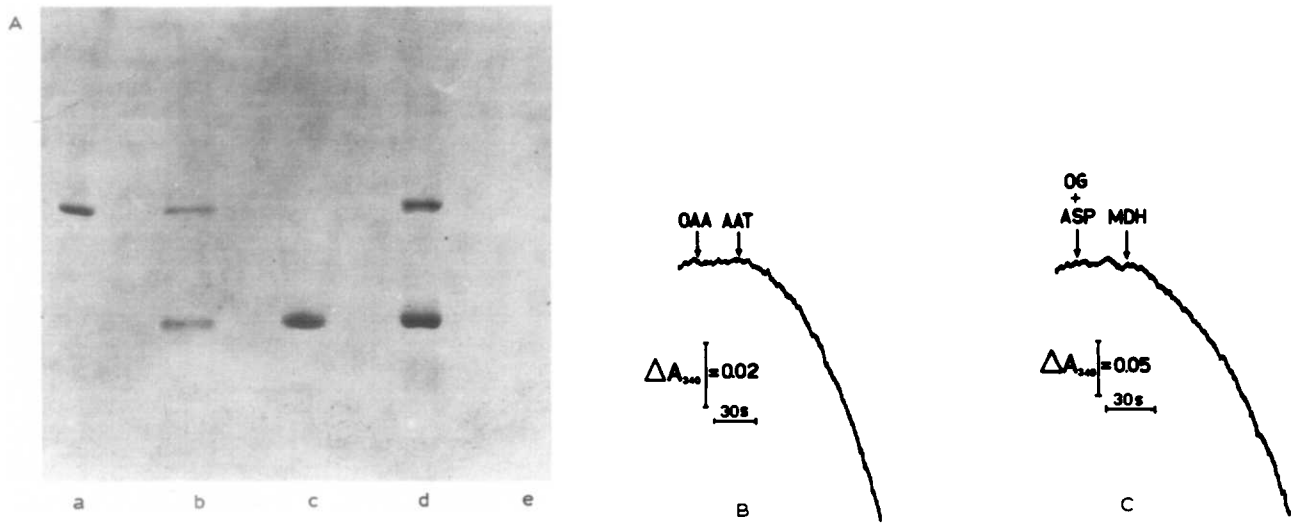

Fig. 1. Specific efflux of enzymes from mitochondria in response to effectors. (A) Demonstration of the phenomenon by SDS-polyacrylamide gel electrophoresis. Track a contained purified mitochondrial aspartate aminotransferase (7.5 μg) and track c contained purified mitochondrial malate dehydrogenase (7.5 μg). Track b contained the supernatant from mitochondria (6.8 mg) incubated with aspartate aminotransferase (25 μg) and track d contained the supernatant from mitochondria (6.8 mg) incubated with malate dehydrogenase (25 μg). Track e was a control and contained the supernatant from mitochondria (6.8 mg) to which no effector had been added. (B and C) Demonstration of the phenomenon by activity measurements. (B) Mitochondria (2 mg) were incubated for 1 min at 25°C in a standard medium consisting of 0.25 M sucrose, 20 mM Tris-HCl (pH 7.25), 1 mM EGTA in the presence of 0.2 mM NADH. Where indicated oxaloacetate (OAA, 2 mM) and aspartate aminotransferase (AAT, 3 μg) were added to the suspension and the decrease in absorbance measured as a function of time as liberated malate dehydrogenase catalyzed oxidation of NADH. (C) Mitochondria (3 mg) were incubated under the experimental conditions reported in B. Where indicated aspartate (ASP, 12.5 mM), 2-oxoglutarate (OG, 3 mM) and malate dehydrogenase (MDH, 3 μg) were added and the decrease in absorbance consequent on oxidation of NADH was measured as a function of time.

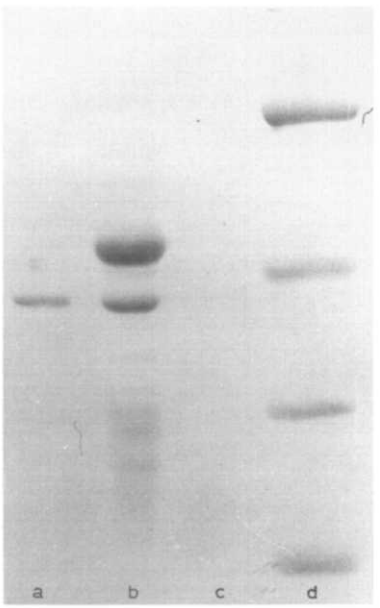

Fig. 2. Identification of aspartate aminotransferase in supernatants from mitochondria treated with malate dehydrogenase by immunoprecipitation. Mitochondria (7.0 mg) were incubated in standard medium containing malate dehydrogenase (12 μg) after which organelles were removed by centrifugation. The supernatant was treated with anti-aspartate aminotranferase antiserum and then with protein A. Analysis of the material absorbed onto protein A is shown in track b. Track a contained purified aspartate aminotranferase (5 μg), track d contained standard proteins, and track c contained supernatant from mitochondria with no added malate dehydrogenase.

presumed to be malate dehydrogenase, that catalysed reduction of oxaloacetate (Fig. 1B).

Addition of malate dehydrogenase to mitochondria caused efflux of an activity presumed to be aspartate aminotransferase, that produced oxaloacetate from the substrate pair as judged by its reduction by NADH (Fig. 1C).

In both cases the rate of oxidation of NADH was not linear but increased at a declining rate as would be expected because of continuing release of intramitochondrial enzyme.

TABLE I

Efflux of aspartate aminotransferase and malate dehydrogenase from mitochondria promoted by the precursor of aspartate aminotransferase

In all cases enzyme activity is expressed in units of change in absorbance at 340 nm for min caused by addition of 20 μl of postmitochondrial supernatant to the cuvette. The controls were from suspensions incubated without added precursor.

Enzyme assayed	Control (ΔA/min)	Test (ΔA/min)
Aspartate aminotransferase	0.012	0.056
Malate dehydrogenase	0.053	0.115
Glutamate dehydrogenase	0.020	0.021
Fumarase	0.000	0.000
Isocitrate dehydrogenase	0.000	0.000

It should also be noted that, in agreement with our previous reports [7] addition of cytosolic aspartate aminotransferase to mitochondria did not result in release of malate dehydrogenase (results not shown in detail).

Further confirmation of the identity of the protein exported from mitochondria by added malate dehydrogenase was provided by the experiment shown in Fig. 2. The supernatant was treated with anti-aspartate aminotransferase antiserum and the immunoprecipitate absorbed onto protein A.

Electrophoresis (Fig. 2) showed a band from the immunoprecipitate (track b) co-migrating with aspartate aminotransferase (track a). Track c contained the supernatant from mitochondria with no effector added and track d contained standard proteins.

Since aspartate aminotransferase is synthesised as a precursor with molecular weight greater than that of the mature form, tests were carried out to ascertain whether precursor uptake by mitochondria is accompanied by efflux of intramitochondrial enzymes. The results are shown in Table I. Small amounts of both aspartate aminotransferase and malate dehydrogenase were found in supernatants from mitochondria suspended in the absence of precursor (controls). However, incubation with the precursor showed a considerable increase in both activities indicative of efflux of enzymes from the organelles. No evidence was found for efflux of glutamate dehydrogenase, fumarase or isocitrate dehydrogenase, thus confirming the specificity of the process.

Kinetics of the release process

The quantity of enzyme released by mitochondria was dependent on the time of incubation with the effector enzyme. Electrophoresis of supernatants from mitochondria incubated with malate dehydrogenase for times between 10 s and 10 min showed increasing amounts of aspartate aminotransferase up to about 5 min, as judged by intensity of staining, after which the amount seemed to plateau. Consistently, a decrease in the residual externally added malate dehydrogenase was also found. Similar results were obtained for release of malate dehydrogenase by aspartate aminotransferase (Figs. 3A and 3C, respectively).

Quantitative measurements of the rates of the efflux process are given in Figs. 3B and 3D. In these experi-

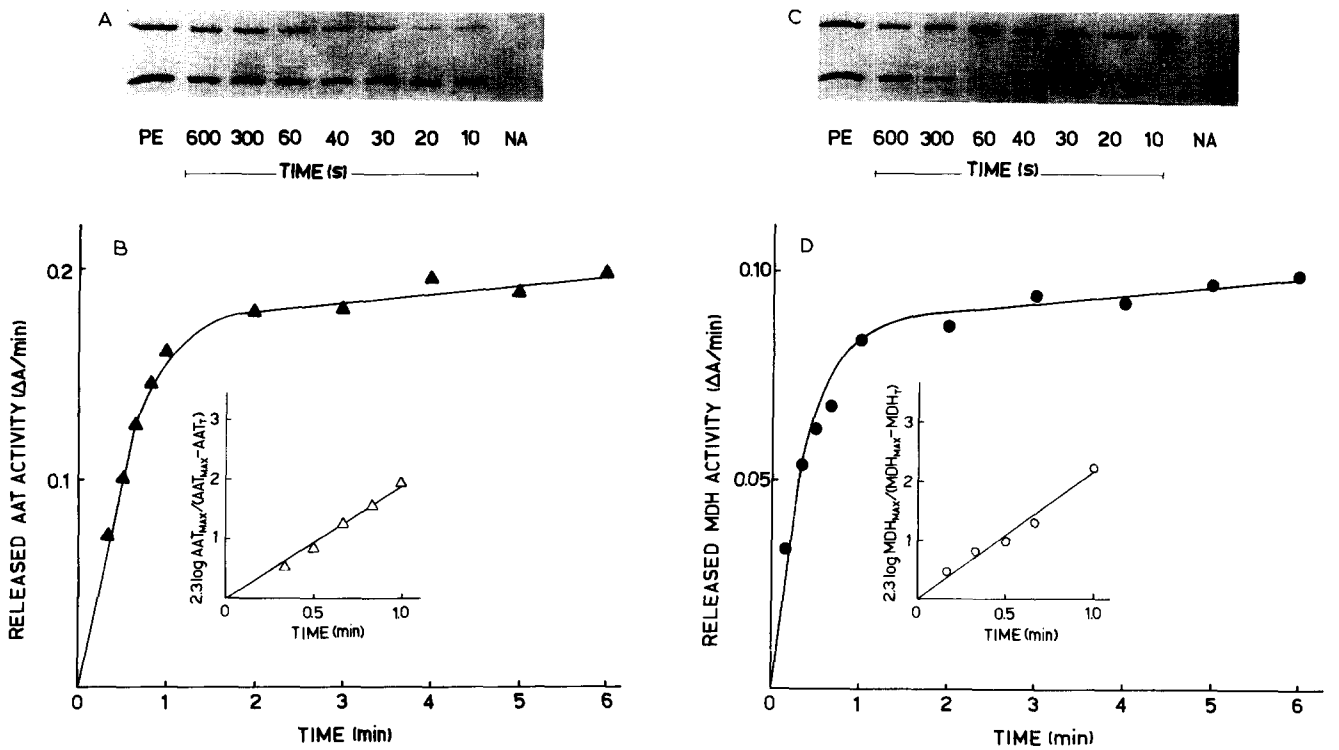

Fig. 3. Rates of release of enzymes from mitochondria. Mitochondria were incubated under standard conditions with effector enzyme for various times. Released enzyme was either subjected to electrophoresis (A and C) or its activity was measured by the usual spectrophotometric method with results expressed as rate of change of absorbance at 340 nm (B and D), insets to the Figs. B and D show the results as log plots. (A and B) Release of aspartate aminotransferase effected by malate dehydrogenase. Malate dehydrogenase (5 and 2 μg/ml in A and B, respectively) was incubated with mitochondria for the indicated times (s) and release of aspartate aminotransferase measured as reported in Methods. PE, purified mitochondrial enzymes: aspartate aminotransferase (up) and malate dehydrogenase (down); NA, no addition. (C and D) Release of malate dehydrogenase effected by aspartate aminotransferase. Aspartate aminotransferase (5 and 1.7 μg/ml in C and D, respectively) was incubated with mitochondria for the indicated times (s) and release of malate dehydrogenase measured as reported in Methods. PE, purified mitochondrial enzymes: aspartate aminotransferase (up) and malate dehydrogenase (down); NA, no addition.

ments supernatants from mitochondria incubated for controlled times with effector were assayed for released enzyme activity under conditions of saturating substrate, that is, where activity is directly proportional to quantity of enzyme. The results are shown in Fig. 3B for release of aspartate aminotransferase and Fig. 3D for release of malate dehydrogenase. In the insets of these graphs the results have been re-plotted as log plots showing that the release process is first order over the initial 1 min period. In both cases the first-order rate constant was of the order of 2 min^{-1} corresponding to a half-life of about 0.35 min. These values are very similar to those for the uptake of the native enzyme into mitochondria [25].

Dependence of rate of efflux on external enzyme concentration

The initial rate of efflux of aspartate aminotransferase was measured as a function of amount of externally added malate dehydrogenase and vice versa.

The results are presented as double-reciprocal plots in Fig. 4B and Fig. 4D, respectively. The amount of externally added aspartate aminotransferase required to elicit half-maximal rate of efflux of malate dehydrogenase was 0.7 μg/ml. In the reciprocal reaction, the amount of malate dehydrogenase required for half-maximal rate of aspartate aminotransferase efflux was 0.3 μg/ml.

In parallel experiments, exported enzymes were detected by gel electrophoresis as a function of amount of externally added effector enzymes (Figs. 4A and 4C) The qualitative results obtained were consistent with the results shown in Figs. 4B and 4D, respectively.

Inhibition of rate of efflux by β-mercaptoethanol and by bathophenanthroline

Given that uptake of aspartate aminotransferase and of malate dehydrogenase into mitochondria are both inhibited by β-mercaptoethanol and by bathophenan-

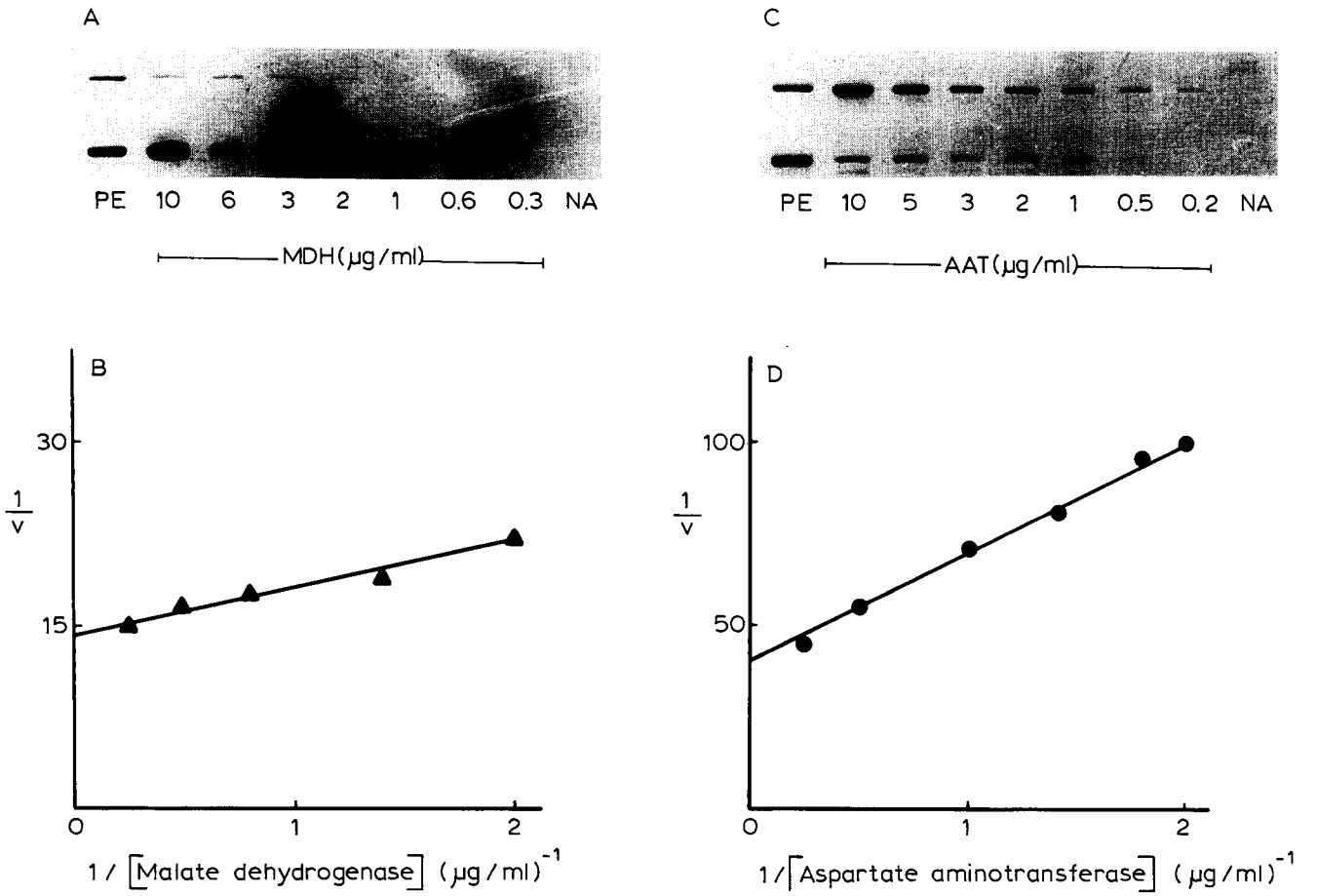

Fig. 4. Dependence of rate of release on concentration of effector enzyme. (A and C) Mitochondria were incubated for 10 s with the indicated increasing amounts of effector enzyme (μg/ml) after which organelles were removed by centrifugation and the supernatants subjected to electrophoresis. In A the effector enzyme was malate dehydrogenase and in C the effector enzyme was aspartate aminotransferase. PE, purified mitochondrial enzymes: aspartate aminotransferase (up) and malate dehydrogenase (down); NA, no addition. (B and D) Mitochondria were incubated as in Fig. 1B and Fig. 1C for 10 s, with varying amounts of effector enzyme (μg/ml). The substrates were added and release activity measured as ΔA/min. V is expressed as activity released in 10 s. In B the effector enzyme was malate dehydrogenase and in D the effector enzyme was aspartate aminotransferase.

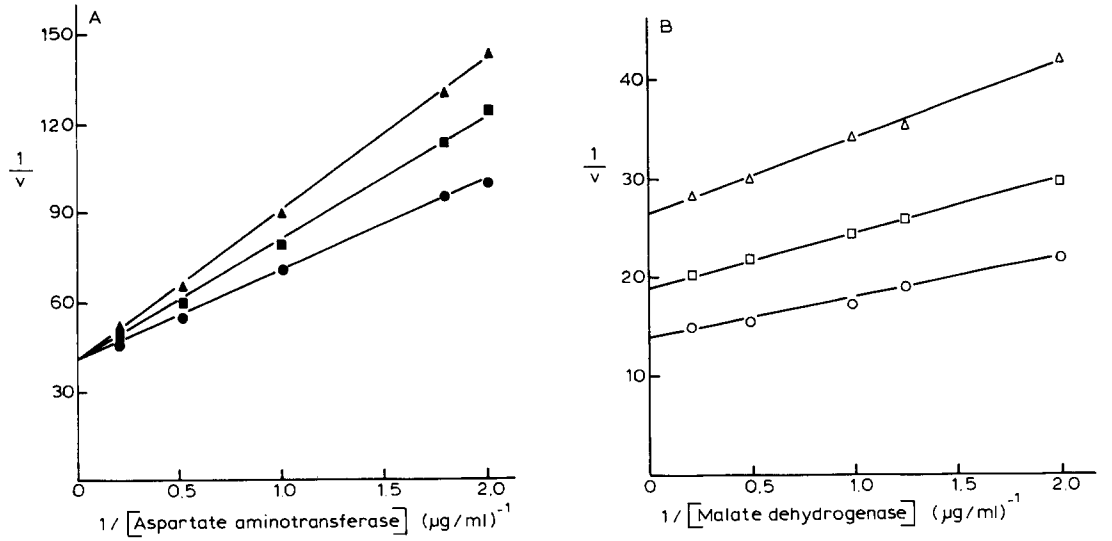

Fig. 5. Inhibition of release by β-mercaptoethanol and by bathophenanthroline. The experiments were carried out as described in Fig. 4, except that, where appropriate, inhibitors were added before addition of effector enzymes. (A) 2 mg of mitochondrial protein were used and the effector enzyme was aspartate aminotransferase. The concentrations of β-mercaptoethanol and bathophenanthroline were 10 μM (▲) and 20 μM (■), respectively. (●) indicates the control reaction with no inhibitors. (B) As for A except that the effector enzyme was malate dehydrogenase and the concentrations of β-mercaptoethanol and bathophenanthroline were 25 μM (△) and 10 μM (□) respectively. (○) indicates the control reaction with no inhibitors.

throline [25,26] it was of interest to test the effects of these compounds on the corresponding efflux processes.

The results are shown in Fig. 5A for export of malate dehydrogenase promoted by aspartate aminotransferase and in Fig. 5B for export of aspartate aminotransferase promoted by malate dehydrogenase. In each case the initial rate of efflux of enzyme was measured as a function of concentration of effector both in the absence or in the presence of the test substances β-mercaptoethanol or bathophenanthroline. The results are shown in Figs. 5A and 5B as double-reciprocal plots. Both substances were inhibitory but whereas they inhibited export of malate dehydrogenase competitively (as shown by the intercept of the lines on the *Y*-axis in Fig. 5A), non-competitive inhibition was found for export of aspartate aminotransferase (Fig. 5B).

In control experiments neither substances at the concentrations used had any effect on the activities of aspartate aminotransferase or malate dehydrogenase.

Energy dependence of the efflux process

Given that the uptake of proteins into mitochondria is an energy-dependent process [2–6], it was of interest to examine this also for efflux. Results of these experiments are shown in Fig. 6A for efflux of malate dehydrogenase and in Fig. 6B for efflux of aspartate aminotransferase. The experimental system used was as described in Figs. 1B and 1C, but with a putative activator or inhibitor added to the mitochondrial suspension before addition of the effector enzyme.

In the case of efflux of malate dehydrogenase caused by external aspartate aminotransferase (Fig. 6A) the process was strongly inhibited by addition of carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and by antimycin, an uncoupler and blocker of electron transport, respectively. The ionophore nigericin also inhibited, as did valinomycin in phosphate medium. On the other hand, valinomycin in a high K^+ medium and the metabolic substrate succinate activated the process as did ATP both alone and in the presence of oligomycin although in the latter case a marked stimulation was observed only when ATP was added before oligomycin. Oligomycin alone was also stimulatory.

Exactly the same effects were observed for efflux of aspartate aminotransferase effected by addition of malate dehydrogenase (Fig. 6B). It is important to note that none of the substances tested had any activatory or inhibitory effect on malate dehydrogenase or aspartate aminotransferase when tested in controls.

In all cases, electrophoretic analysis of supernatants was used as a qualitative confirmation of the rate measurements (Fig. 7). Interestingly comparison made of the intensity of staining of the bands shows a good correspondence between the residual amounts of the effector enzymes and the amounts of the effluxed proteins; for instance, the activation of AAT efflux by oligomycin causes a decrease of the residual MDH (compare in Figs. 7B and 7D, lanes O and C). This further indicates that uptake of externally added enzymes correlates with the efflux of intramitochondrial proteins (see also Figs. 3A and 3C).

Comparison of the catalytic properties of native and exported enzymes

Interesting differences were observed between native and exported enzymes in terms of dependence of activ-

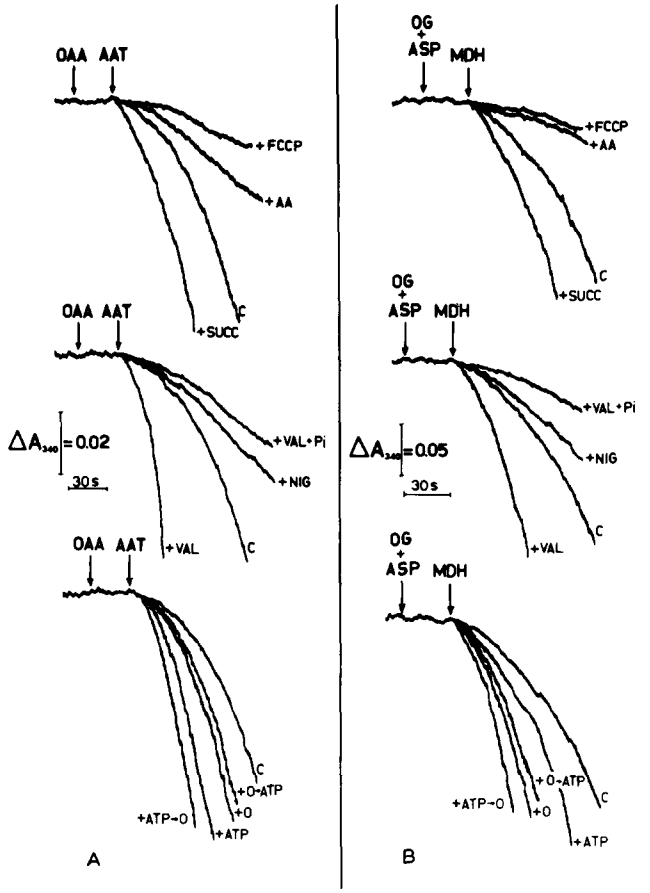

Fig. 6. Energy dependence of the release process determinated by activity measuraments. The experimental design was essentially the same as in Fig. 1B and Fig. 1C except that the test substance was added 1 min before addition of effector enzyme. When either valinomycin or nigericin were used reaction medium was the following: 100 mM KCl 20 mM Tris-HCl (pH 7.25), 1 mM EDTA-Tris. Added substances were: carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 1 μM), antimycin (AA, 1 μg), succinate (SUCC, 2 mM), nigericin (NIG, 1 nmol), valinomycin alone (VAL, 0.05 μg) or in the presence of phosphate (VAL + P_i, 0.05 μg and 2.5 mM, respectively), oligomycin (O, 0.2 μg) either alone or together with ATP (1 mM) (in any case time interval between the two additions was 1 min). The curve labelled C is a control with no added test substance.(A) The amount of mitochondrial protein was 2 mg and the effector enzyme was aspartate aminotransferase (3 μg). (B) The amount of mitochondrial protein was 2 mg and the effector enzyme was malate dehydrogenase (3 μg).

ity on pH. These are shown in Fig. 8A for malate dehydrogenase and in Fig. 8B for aspartate aminotransferase.

In the case of malate dehydrogenase (Fig. 8A) the difference occurred in the pH range 6 to 8 where the native enzyme showed a continuous increase of activity from low to high pH whereas the exported form showed a small but reproducible peak in activity at about pH 6.8.

The difference in pH profile was much more marked in the case of aspartate aminotransferase (Fig. 8B). At high pH (7 to 9) the activity of the exported form decreased continuously whereas that for the native enzyme increased.

It should be noted that the behaviour observed for the native enzyme was the same whether purified enzymes were used or whether activity was obtained by lysis of freshly prepared mitochondria with Triton X-100.

Discussion

The results reported here confirm our previous observation [15,16] that incubation of mitochondria with malate dehydrogenase causes efflux of aspartate aminotransferase activity and vice versa, but have the added dimension that electrophoretic analysis of the post-mitochondrial supernatants allowed direct demonstration of efflux of proteins of the expected sizes. Moreover,electrophoresis experiments show that any decrease in the intensity of staining of the residual effector enzymes corresponds to an increase of the effluxed proteins, thus clearly indicating that uptake of externally added protein is actually accompanied with release of intramitochondrial polypeptides. The results also give further evidence for the specificity of the process in that on incubation of mitochondria with malate dehydrogenase the only protein to appear in the supernatant (with the exception of residual malate dehydrogenase) had the size expected for aspartate aminotransferase. Moreover, this protein could be immunoprecipitated with anti-aspartate aminotransferase antiserum thus confirming its identity (Fig. 2). Similarly incubation of mitochondria with aspartate aminotransferase released only protein of the expected molecular weight for malate dehydrogenase.

It should be noted that our observation of movement of proteins across mitochondrial membranes is not unique. Waksman and his colleagues [28] have shown that aspartate aminotransferase can translocate from the matrix into the intermembranal space in response to low-molecular weight effectors or changes in the energetic state of the organelles, but the relationship between those phenomena and the ones reported here is unclear. On the other hand, Miralles and co-workers [14] have demonstrated release of cytochrome *c* from mitochondria incubated with apoprotein, a phenomenon which seems to be directly related to that reported here (except, of course, that cytochrome *c* has to transverse only the outer membrane to be released from mitochondria).

The characteristics of the release of aspartate aminotransferase and malate dehydrogenase from mitochondria parallel very closely those of the uptake of the same enzymes into organelles, a process that we have previously studied in detail [26,27,29,30]. The efflux is fast, having a half-life of about 0.35 min (Fig. 3), and shows saturation kinetics (Fig. 4). The latter observation

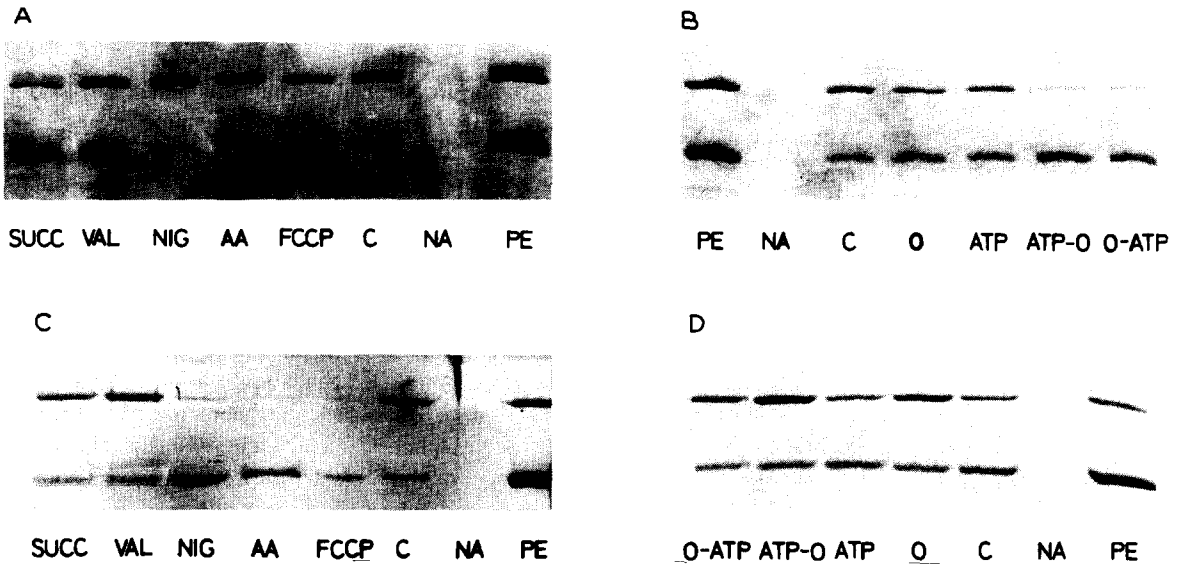

Fig. 7. Energy dependence of the release process determinated by electrophoresis. The results shown correspond to those reported in Fig. 6 except that post mitochondrial supernatant were subjected to electrophoresis. (A and B) Release of aspartate aminotransferase effected by malate dehydrogenase (3 μg/ml). (C and D) Release of malate dehydrogenase effected by aspartate aminotransferase (3 μg/ml). C, control with no added test substance; SUCC, succinate; VAL, valinomycin; NIG, nigericin; AA, antimycin A; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; O, oligomycin, ATP, adenosine triphosphate; NA, no addition; PE, purified mitochondrial aspartate aminotransferase and malate dehydrogenase.

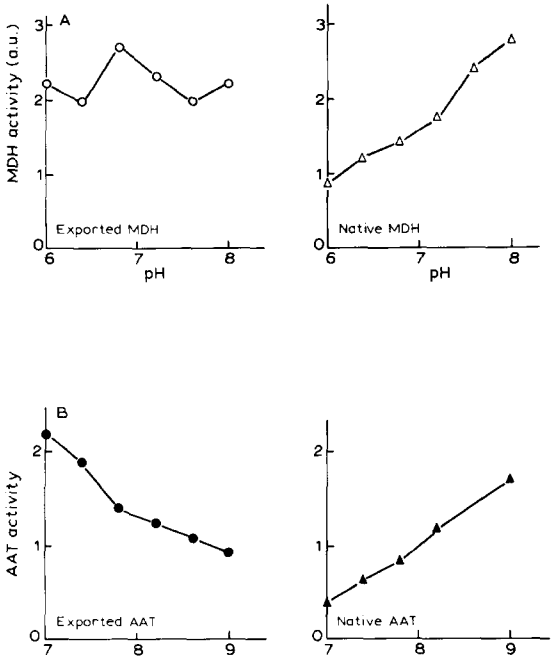

Fig. 8. Dependence of catalytic activity on pH for exported and native enzymes. Enzyme was exported by incubation of mitochondria (2 mg) in standard medium (1 ml) with effector enzyme (5 μg) for 6 min. Mitochondria were removed by centrifugation and ruptured by addition of Triton X-100 (10%, v/v, 100 μl) to liberate native enzymes. Alternatively, purified enzymes were used with identical results. Aliquots (100 μl) of post-mitochondrial supernatants were assayed by the usual spectrophotometric methods; 10 μl of Triton X-100 (10%, v/v) was assayed to maintain conditions identical with those used to assay mitochondrial lysates. Aliquots (10 μl) of lysated were assayed. The buffer used for the assays was 0.25 M phosphate adjusted to the required pH by addition of NaOH. (A) Malate dehydrogenase, (B) aspartate aminotransferase.

argues strongly for involvement of a receptor in the inner membrane system. Further evidence for this, and a demonstration of the intimate link between uptake and export, is provided by the inhibitory effects of β-mercaptoethanol and bathophenanthroline (Fig. 5). We have previously shown that uptake of aspartate aminotransferase into mitochondria is competitively inhibited by β-mercaptoethanol [26] and by bathophenanthroline [27]; precisely the same inhibitory effects were observed for efflux of malate dehydrogenase promoted by aspartate aminotransferase (Fig. 5A). Similarly uptake of malate dehydrogenase into mitochondria is non-competitively inhibited by β-mercaptoethanol [26] and by bathophenanthroline [27], a situation mirrored by the effects of these substances on export of aspartate aminotransferase promoted by malate dehydrogenase (Fig. 5B).

A final demonstration of the connection between uptake and release is provided by the energy dependence of the two processes (Fig. 6). The uncoupler FCCP and an inhibitor of electron transport, antimycin, both reduce efflux dramatically. On the other hand energization of mitochondria with succinate or with ATP stimulates the process. The effects of ionophores was particularly interesting. Valinomycin which, in the presence of KCl, dissipates the mitochondrial transmembrane potential whilst increasing ΔpH [30] was strongly activatory. Nigericin, on the other hand, which collapses ΔpH [31] was strongly inhibitory. These results point very strongly to the transmembrane pH gradient as the driving force in protein efflux from mitochondria (but see below). The observed effect of

oligomycin and ATP is consistent with this but the reason for the different effects seen, depending on the order of addition of the two substances, merits some comment. The fact that oligomycin, an ATPase inhibitor, added before addition of ATP results in no activation, whereas its addition after ATP does lead to activation, indicates that the ATP effect arises from its hydrolysis and consequent generation of an ion gradient, rather than from phosphorylation of either enzymes themselves or any component of the mitochondrial membrane system. This is again what is observed for the corresponding uptake processes [26,27].

An obvious question arising from the results presented here is, does incubation of mitochondria with aspartate aminotransferase cause efflux of aspartate aminotransferase as well as malate dehydrogenase? The techniques used in the majority of this work do not allow that question to be answered directly since residual effector enzyme in the incubation medium would prevent detection of released enzyme of the same species. In a separate experiment, however, it was demonstrated that incubation of mitochondria with the precursor form of aspartate aminotransferase caused efflux of both aspartate aminotransferase and malate dehydrogenase under conditions where the precursor is known to be imported into the organelles and processed.

In this latter experiment (Table I), interpretation of activity measurements for aspartate aminotransferase is complicated by uncertainty as to whether the precursor form is catalytically active or inactive [32,33]. However, given that under the conditions used the majority of the precursor form is taken up into the organelles [21], only a small part, if any, of the enzyme activity detected in the postmitochondrial supernatant could have been due to residual precursor. The question also arises as to why incubation of aspartate aminotransferase with mitochondria releases malate dehydrogenase. The answer to this must relate to our previous observation [26] that these two enzymes share a common receptor for uptake into the organelles and suggests that uptake and efflux are both functions of the same mitochondrial apparatus. Indeed, the very close similarity between the rates of uptake and efflux, their kinectis of inhibition by β-mercaptoethanol and by bathophenanthroline, their identical energy requirements, and the evidence that uptake of the effector enzymes is observed together with the appearance of effluxed proteins (see above) suggest that the processes are coupled; that is, there may be an exchange of one protein molecule coming out for each one going in. Attempts are being made to measure the stoichiometry of uptake and efflux to gain evidence on this point.

While more work is required to establish the full biological significance of the efflux process reported here, particular note should be taken of the fact that exported enzymes show different kinetic characteristics from those of the native form (Fig. 8). This, combined with the fact [35] that mitochondrial aspartate aminotransferase in rat liver is degraded in the lysosomes, suggests that the modified exported enzymes may represent molecules on a pathway to degradation.

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