Direct Evidence for Internalization of Mitochondrial Aspartate Aminotransferase into Mitoplasts†

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ABSTRACT: Mitochondrial aspartate aminotransferase, an enzyme localized on the inner face of the inner mitochondrial membrane, is released into the intermembrane space upon addition of a "movement effector" (succinate, fumarate, pyruvate, or glutamate) [Waksman, A., & Rendon, A. (1974) *Biochimie 56*, 907–924]. After removal of the movement effector, 90% of the released enzyme rebound to mitoplasts. Lubrol fractionation showed that this bound activity was associated with the inner membrane. Internalization was demonstrated by using both enzymatic and molecular approaches. It was found that 70% of the reassociated enzyme became inaccessible from the outside of the mitoplast either to a nonpermeating substrate (NADH), to mild protease hydrolysis, or to recognition by a specific antibody. In contrast, in inside-out vesicles, the enzyme remained accessible to NADH, protease, and antibodies. Latency measurements performed at different temperatures on whole intact mitochondria confirmed the existence of reversible intermembrane movement of the enzyme in situ.

The lateral movement of membrane components is generally known to occur. Moreover, recent studies provide evidence for possible transverse movements of membrane proteins and lipids (Costa et al., 1976; Little & Widnell, 1975; De Kruijff & Baken, 1978; Rothman & Kennedy, 1977). These spatial phenomena could be of critical importance for the temporal organization, biogenesis, and functional properties of biological membranes. The observation that proteins of both inner mitochondrial membrane and matrix are synthetized principally in the cytoplasm further supports the possibility for transmembrane movement of proteins (Schatz & Mason, 1974).

In earlier reports, Waksman & Rendon (1974), Crêmel et al. (1976), and Waksman et al. (1977) demonstrated the existence of intramitochondrial large-amplitude protein movements, movements which were shown to be reversible, specific, and dependent upon the nature of the membrane lipids. In the course of these studies, it became clear that reversible translocation of some proteins through the inner mitochondrial membrane should occur. Using fractionation methods, latency measurements, and accessibility to protease or to a specific antibody, we have demonstrated the internalization of mitochondrial aspartate aminotransferase (AAT)[1] into isolated mitoplasts or into the matrix of intact mitochondria.

Materials and Methods

Lubrol WX and Triton X-100 were from Sigma Chemical Co. (St Louis, MO). Digitonin, trypsin, and chymotrypsin were from Merck (Darmstadt, West Germany). Iodine-125 was obtained from the Radiochemical Center (Amersham, U.K.). Centrifugations were generally performed in a Spinco Model L 50 or in a MSE Hi-Spin 21 centrifuge. All manipulations were performed at 4 °C unless otherwise stated.

Preparation of Mitochondria and Mitochondrial Fractions. Rat liver mitochondria were prepared in 0.25 M sucrose, according to Harel et al. (1957) as modified by Waksman & Rendon (1974), except that mitochondria were only washed twice. For the preparation of mitoplasts (inner membrane + matrix complex) and intermembrane fluids, the digitonin method of Levy et al. (1967) and Schnaitman et al. (1967), as modified by Waksman & Rendon (1974), was used. For further separation of inner membrane from the matrix, the Lubrol technique described by Chan et al. (1970) was employed.

Submitochondrial particles (SMP) were prepared according to Muscatello & Carafoli (1969). Mitochondrial protein (50 mg/mL) was used for this purpose, with sonication performed on 4 mL at a time with an MSE 150-type sonifier, for two 30-s periods at 0 °C. The partially broken and unbroken mitochondria were separated by centrifugation at 8000*g* for 10 min. The supernatant fraction containing the particles was then centrifuged at 100000*g* for 45 min. The pellet was resuspended in 4 mL of 0.25 M sucrose. In such preparations, 80% of the particles was inside out, according to the criteria of respiration in the presence of exogenous cytochrome *c* (Muscatello & Carafoli, 1969; Chance et al., 1970) and latency of aspartate aminotransferase (Scholte et al., 1973).

Incubations Leading to Release and Reassociation[2] of AAT from and to Submitochondrial Fractions. AAT enriched intermembrane fluid was prepared by incubation of mitochondria with succinate prior to digitonin treatment, as described by Waksman & Rendon (1974). This fluid was concentrated 10 times in an Amicon cell fitted with a Diaflo membrane PM 30 and dialyzed against 3 L of 0.25 M sucrose in a hollow fiber mini beaker (Dow Chemical) for 4 h at 4 °C to remove dialyzable molecules, including succinate. This fraction was then centrifuged for 15 min at 100000*g* to remove precipitated material.

For reassociation or "internalization", 0.5 mL of mitoplasts (33 mg of protein per mL) that had been freshly isolated from succinate (50 mM) incubated mitochondria was then incubated with 0.5 mL of the dialyzed, concentrated intermembrane fluid (11 mg of protein per mL), prepared as described above.

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[1] Abbreviations used: AAT, L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1); NADH, reduced nicotinamide adenine dinucleotide; SMP, submitochondrial particles, i.e., inside-out vesicles.

[2] "Release" denotes the movement of AAT toward the intermembrane space, movement which is induced by a "movement effector" (succinate). "Reassociation" and "rebinding" are both general expressions related to experimental procedure. "Internalization" describes rebinding resulting in inaccessibility on the outer surface of a vesicle.

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Incubations were performed for 5 min at 37 °C, and mitoplasts were separated from the supernatant fraction by 20-min centrifugation at 30000*g*. The pellets were resuspended in 1 mL of 0.25 M sucrose.

In the case of SMP and for release experiments, 1 mL of sonicated material (5 mg of protein per mL) was incubated under the same conditions as for mitochondria. At the end of the incubation, the mixture was centrifuged for 45 min at 100000*g* to separate particles from the supernatant containing released proteins. The latter was dialyzed to eliminate succinate and concentrated as the intermembrane fluid. Reassociation was performed as for the internalization incubation with mitoplasts, except that the final pellets were collected at 100000*g* for 45 min. These pellets were resuspended in 3 mL of 0.25 M sucrose.

Enzymatic Assay and AAT Latency. AAT activity was determined according to the coupled method of Karmen (1955). For measurement of total activity in membrane fractions, 0.1% Triton X-100 was added to the medium.

For measurements of latency of AAT activity, all solutions were prepared in 0.25 M sucrose to preserve isoosmolarity. The Karmen medium was modified to contain 10 mM sodium phosphate buffer at pH 7.0, 0.8 mM sodium aspartate, and 0.16 mM sodium 2-oxoglutarate. Latent activity was revealed by addition of 0.1% Lubrol WX or Triton X-100. Measurements were performed on sucrose-washed mitoplasts. Similar experiments were performed on whole mitochondria in the absence of detergent and in the presence of succinate.

Protein Determination. Protein was assayed according to Lowry et al. (1951), with bovine serum albumin as standard.

Aspartate Aminotransferase Purification. Mitochondrial AAT was purified by a two-step procedure as described by Crêmel (1977).

AAT was released in the intermembrane space by succinate treatment. The corresponding fluid was isolated and applied to an affinity chromatography column of activated Sepharose 4B with a six-carbon side arm coupled with aspartate. After elution, the active peak was rechromatographed on a Sephadex CM-50 ion-exchange column. This preparation showed excellent homogeneity on polyacrylamide gel electrophoresis, isoelectrophoresis, and immunoelectrophoresis.

Preparation of Antiaspartate Aminotransferase Antibody (Anti-AAT). For the preparation of antiserum, young adult rabbits were injected in the footpads weekly for 3 weeks with 0.5 mL of a purified mitochondrial AAT prepared as described above (1 mg of protein per mL) supplemented with an equal volumn of Freund's adjuvant (Difco Laboratories, Detroit, MI). Three weeks after the last injection, 1 mL of the purified enzyme preparation was injected intravenously. The animals were bled from the ear, about 20 mL of blood being withdrawn on 3 successive days into sterile tubes. The crude globulin fraction from the sera obtained by low-speed centrifugation was precipitated with 50% saturated ammonium sulfate. After 1-h equilibration at 4 °C and centrifugation for 15 min at 2500*g*, the pellet was dissolved in 10 mM phosphate buffer at pH 7.0 to one-third of the volume of the serum from which the globulins were obtained. This antiserum was found to be monospecific for AAT both by double immunodiffusion and by immunoelectrophoresis. Control sera were obtained from the same animals before injection of the antigen and were prepared in a similar manner.

Iodination Procedure. The method described by Greenwood et al. (1963) was used. To 20 μg of protein, 1 mCi of ^{125}I was added in 50 μL of the appropriate buffer at pH 7.0. Chloramine-T (50 μg) in 10 μL of buffer was added to start the reaction. After a 30-s incubation at 4 °C, the reaction was stopped by the successive additions of 200 μg of sodium metabisulfite in 20 μL of buffer and 500 μg of KI in 50 μL of the same buffer. The iodinated proteins were separated from the mixture on a Sephadex G-25 column (10 × 1 cm) and eluted with the initial buffer. This procedure was applied to AAT eluted from the affinity chromatography column and to antisera, obtained as described above.

Immunoprecipitation of AAT Solubilized from Mitoplasts. For immunoprecipitation, proteins were extracted by sonication from sucrose-incubated (control), succinate-treated, and internalized mitoplasts previously suspended in 10 mM phosphate buffer at pH 7.0. They were sonicated for six 30-s periods at 4 °C in a MSE sonicator. The samples were centrifuged for 60 min at 100000*g*. The supernatant fraction containing 80% of the total protein and 90% of the total AAT activity was used for the immunoprecipitation. Both specific and control antisera were iodinated according to the method described for AAT. Final specific activities of the sera were adjusted to 660000 cpm/mg of protein. The approximate equivalence precipitation zone was first determined, and then various amounts of the supernatants described above, ranging from 0.5 to 5 mg of protein, were added to 40 μL of antiserum or control serum (9 mg/mL). The final volume was adjusted to 3 mL with 0.03 M phosphate buffer, pH 7.0, 0.5% Triton X-100, and merthiolate 1/10000. The samples were incubated for 10 min in a shaking bath at 37 °C, allowed to equilibrate to room temperature for 3 h, and finally left to precipitate at 4 °C for 120 h. The precipitates were recovered by centrifugation at 2500*g* for 30 min at 4 °C in a Janetzki T32D centrifuge. The pellets were washed 5 times with 3 mL of buffer, and the samples were counted in an Abott autologic-type γ counter. Precipitation with control serum never exceeded 10% of that of the specific immunoglobulin tests.

Labeled Immunoglobulin Binding Assay. To measure the accessibility of AAT to the specific antibody on the surface of mitoplasts and SMP, the binding method described by Chan & Tracy (1978) was used. Assays were performed on sucrose-incubated and internalized mitoplasts with sucrose-prepared SMP as the control. The final suspension of these samples was made in "H" medium (70 mM sucrose, 220 mM mannitol, 2 mM Hepes, and 0.5 mg/mL bovine serum albumin, pH 7.4, adjusted with KOH). Initially, 10 mg of submitochondrial fractions was incubated with 1.5 mL of normal rabbit immunoglobulin (control serum; 26 mg/mL of protein) in H medium for 20 min at 0 °C. This eliminated nonspecific membrane binding. After centrifugation (30000*g* for 20 min for the mitoplasts; 100000*g* for 30 min for the SMP), the pellets were homogenized and resuspended in the initial volume of H medium. The suspension (400 μL) was then incubated for 20 min at 0 °C with 100 μL of ^{125}I-labeled specific anti-AAT immunoglobulin (50 mg/mL; 35000 cpm/mg). Samples were then centrifuged at the appropriate speeds and washed once in H medium. Pellets were counted in an Abott γ counter.

Measure of Accessibility of Reassociated ^{125}I-Labeled AAT by Limited Protease Treatment. Reassociated mitoplasts and SMP were prepared as described above, except that ^{125}I-labeled AAT was added to the dialyzed fluid (50000 cpm/mg of protein). Mitoplasts were washed once in 0.25 M sucrose, and the pellets were homogenized and resuspended in sucrose (final protein concentration 13 mg/mL). To 10 mg of these submitochondrial fractions (about 13000 cpm/mg of protein), 100 μg of both trypsin and chymotrypsin (1 mg/mL) in 0.25 M sucrose was added. Samples were allowed to incubate at

0 °C for 30 min and were then centrifuged at appropriate speeds (mitoplasts at 30000*g* for 20 min and submitochondrial particles at 100000*g* for 30 min). The supernatants and the pellets were counted in an Abott autologic-type γ counter. The protease activity was controlled on soluble AAT. Under these conditions, 50% of the initial AAT activity was shown to be lost. The limited character of this protease treatment was controlled on mitoplasts by its action on cytochrome *c* oxidase activity. Assays were performed according to the method of Sanadi & Jacobs (1967), by using TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) as the electron carrier. The decrease of cytochrome oxidase activity did not exceed 15% under our experimental conditions, and no activity was released from the membrane during incubation.

Results

Release (externalization) of aspartate aminotransferase (AAT) from mitoplasts into intermembrane fluid is induced by movement effectors such as succinate, fumarate, or pyruvate. Rebinding of the released enzyme to the mitoplasts occurs upon removal of the movement effector (Waksman & Rendon, 1974; Waksman et al., 1977). As AAT was shown to be localized on the inner surface of the inner mitochondrial membrane in sucrose medium (Scholte et al., 1973; Wit-Peeters et al., 1971), reversibility of release implies internalization of the externalized enzyme.

To study this point further, different approaches have been used. In the common part of these experiments, mitochondria were first exposed to succinate, and then intermembrane fluid and mitoplasts were separated after digitonin treatment. The isolated intermembrane fluid was dialyzed to remove succinate and concentrated. This fluid was then reincubated with freshly prepared succinate-treated mitoplasts. SMP (inside-out vesicles) were submitted to a similar procedure. Localization and accessibility of reassociated AAT were assayed by different methods, some related to enzymatic activity and others being molecular approaches. Sucrose-incubated and succinate-treated submitochondrial fractions were used as appropriate controls.

Localization of AAT as Determined by Lubrol Treatment. Lubrol WX is currently used to separate matrix from inner membrane components in mitochondria, so mitoplasts were treated with Lubrol before and after internalization. Succinate was added to an internalized mitoplast fraction in the course of this treatment. Figure 1 and Scheme I show that before internalization, 52% of the total AAT activity was found in the dialyzed intermembrane fluid. From the activity associated with the succinate-treated mitoplast, about 5% was washable by 0.25 M sucrose, 8% was separated in "matrix", and 87% was localized within the inner membrane in 0.25 M sucrose. After internalization, 90% of the total enzyme activity was recovered within the internalized mitoplast. Of this activity 18.5% was washable by sucrose, 4.5% was recovered in matrix, and 77% was found associated to the inner membrane in 0.25 M sucrose. This membrane fraction represented 69% of the enzyme activity rebound to the mitoplast. However, if in the course of Lubrol treatment 5 mM succinate was added, the repartition was now 21% sucrose-washable enzyme activity, 4% membrane-bound enzyme activity, and 75% matrix (Lubrol-soluble) enzyme activity. These results thus show a high binding of AAT to the mitochondrial inner membrane. This rebound enzyme was not solubilized by Lubrol but could be released by succinate treatment and be defined under such conditions as "matrix".

Internalization of AAT Determined by the Accessibility of the Enzyme to NADH. The previous results suggested AAT

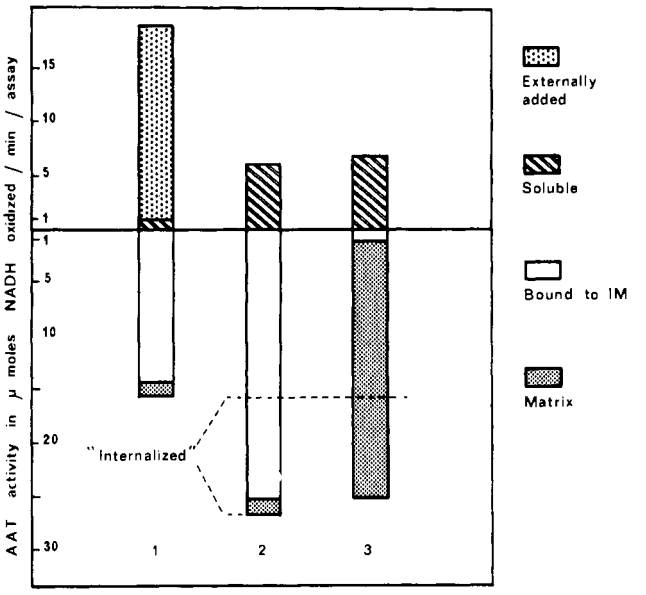
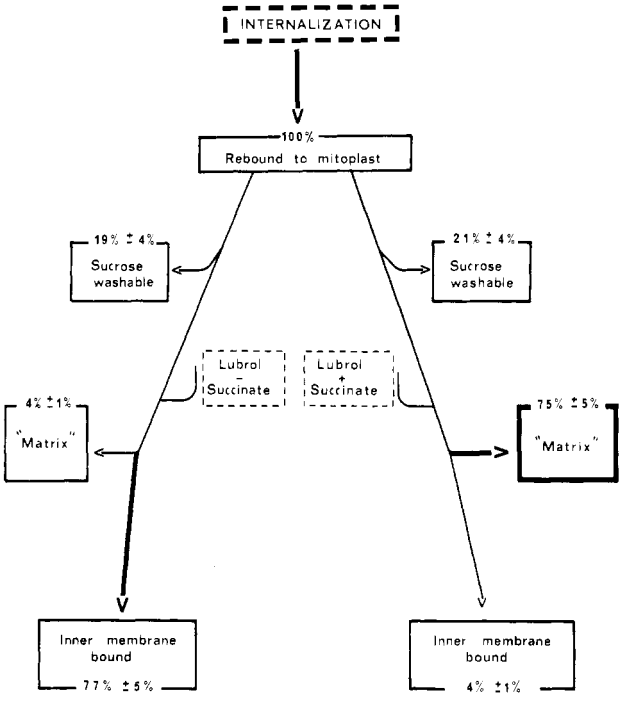


FIGURE 1: Internalization into mitoplasts of mitochondrial aspartate aminotransferase (AAT) as measured by the Lubrol fractionation method. (1) AAT activity per sample before mixing succinate-treated mitoplasts with the corresponding dialyzed intermembrane fluid (see Materials and Methods). Upper dotted area: intermembrane fluid. Upper hatched area: sucrose-washable or released. Lower white area: inner membrane after Lubrol treatment. Lower dotted area: matrix after Lubrol treatment. (2) AAT in isolated mitoplasts after reassociation. (3) The same as described in (2) but in the presence of 5 mM succinate in the course of Lubrol treatment. AAT activity was measured as micromoles of NADH oxidized per minute per assay (mean values of six experiments).

Scheme I: AAT Accessibility in Internalized Mitoplasts after Internalization as Measured by the Lubrol Method[a]



[a] Left-side arrow: Lubrol treatment in the presence of 0.25 M sucrose alone. Right-side arrow: Lubrol treatment in the presence of 0.25 M sucrose supplemented with 5 mM succinate (mean and SEM values of six experiments).

internalization. Further evidence was provided by a study of the accessibility of the reassociated enzyme to substrates to which the inner mitochondrial membrane is impermeable. Inner mitochondrial membrane is well-known to be im-

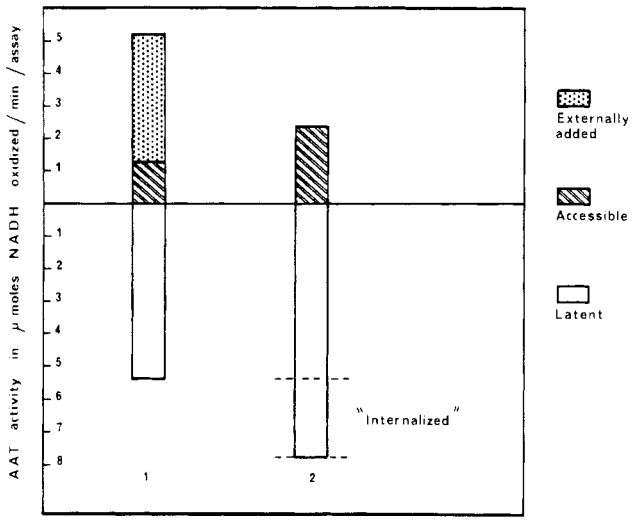


FIGURE 2: Internalization into mitoplasts of mitochondrial aspartate aminotransferase (AAT) as measured by the latency method. (1) Before mixing succinate-treated mitoplasts with the corresponding concentrated and dialyzed intermembrane fluid (see Materials and Methods). Upper dotted area: AAT activity in intermembrane fluid. Upper hatched area: nonlatent AAT activity (accessible on intact mitoplasts). Lower white area: latent AAT activity. (2) AAT latency in isolated mitoplasts after mixing intermembrane fluid with mitoplasts. Latency was measured on intact mitoplasts and Lubrol- or Triton-treated organelles. Activity is expressed in micromoles of NADH oxidized per minute per assay (mean values of four experiments).

permeable to NADH (Lehninger, 1951; Purvis & Lowenstein, 1961). This observation has been confirmed under our experimental conditions. Thus, an enzyme located within the mitoplast and requiring NADH would not be detectable in an intact organelle but should become accessible after solubilization of this organelle by a detergent (latency measurement). We measured AAT activity by a coupled method using NADH as the cofactor. In intact mitoplasts, only a small part of this activity is detectable as compared with detergent-treated organelles. Figure 2 shows that before internalization, 40% of the total AAT activity was in the dialyzed fluid. Of the amount in the mitoplasts, 20% was accessible to direct NADH measurement. After incubation, 89% of the exogenous enzyme rebound to the mitoplast, and of the total activity, 24% was accessible to NADH and 76% remained latent. Expressed as a percentage of the rebound activity, 68% of the exogenous enzyme became latent, compared with 69% of the enzyme found localized within the inner membrane by Lubrol fractionation.

AAT Release from and Rebinding to Submitochondrial Particles (SMP). If AAT is internalized into mitoplasts after succinate removal, SMP (inside-out vesicles) would bind the enzyme without internalization. Release and binding of AAT was assayed with SMP under the same conditions as described for mitoplasts. The specific activity of the enzyme in these vesicles is about 50% lower than in mitoplasts. In SMP isolated in 0.25 M sucrose, 82% of the AAT is readily accessible to exogenous NADH (nonlatent). Such particles released half of their activity in the presence of 5 mM succinate. Reincubation of AAT depleted particles in the presence of dialyzed and concentrated supernatant obtained from such vesicles resulted in the binding of 80% of the added activity (Figure 3A). None of the added activity became latent (Figure 3B); in contrast to the findings with mitoplasts, the AAT which reassociated to SMP became totally accessible to NADH.

Total AAT Measurement by Immunoprecipitation. Although the experiments above indicated that after internalization the major part of the enzyme activity became inaccessible from the outside of the inner mitochondrial membrane, it did not completely exclude the possibility of enzyme activation, although this appeared unlikely as 90% of initial activity was recovered. However, to completely overcome this objection, immunoprecipitation tests were performed with antibodies against purified AAT. Sucrose-incubated, succinate-treated, and internalized mitoplasts were thoroughly sonicated to solubilize the enzyme, and ^{125}I-labeled immune and control sera were used for precipitation. At equivalence points, the specific radioactivity of the precipitates yielded the following results: 92 μg of antibody was precipitated per mg of protein (sucrose-treated mitoplasts), 42 μg/mg was precipitated (succinate-treated mitoplasts), and 72 μg/mg was precipitated (internalized mitoplasts). If we assume the amount of enzyme released from the sonicated sucrose-treated mitoplasts (control) as 100%, then the amount of AAT remaining in succinate-treated mitoplasts is 46%, and the amount

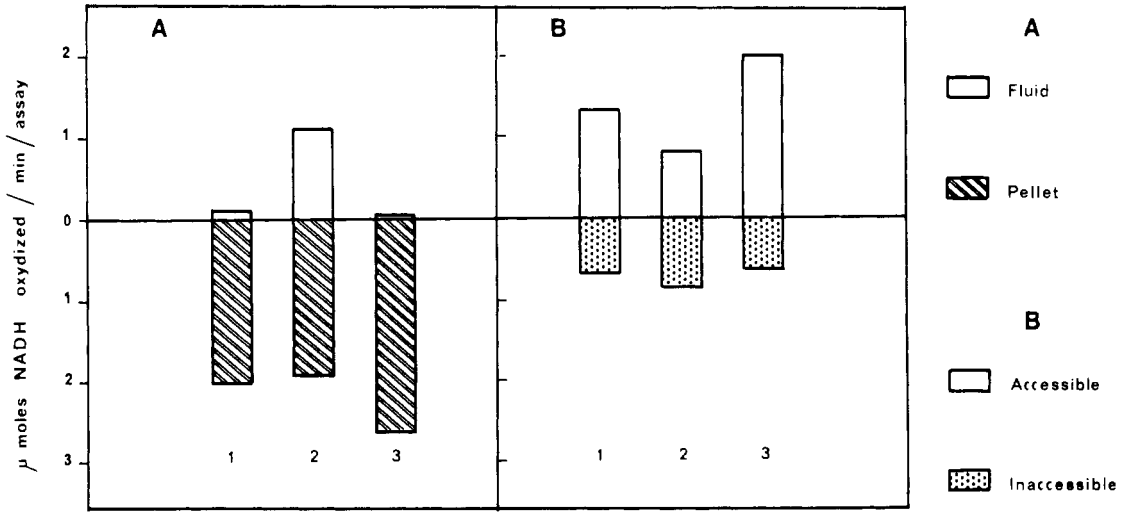


FIGURE 3: Rebinding (A) and latency (B) of AAT activity in submitochondrial particles (SMP). (A) Rebinding of AAT (see Materials and Methods). (B) Latency of AAT after mixing SMP with the incubation medium (see Materials and Methods). Upper white area: sucrose-washable AAT activity or activity accessible to NADH. Lower hatched area: vesicle-bound AAT activity. Lower dotted area: AAT activity inaccessible to NADH. (1) AAT activity in succinate-treated SMP. (2) AAT activity after incubation with dialyzed fluid + 5 mM succinate. (3) AAT activity after incubation with dialyzed fluid, without succinate. Activities are expressed in micromoles of NADH oxidized per minute per assay (mean values of three experiments).

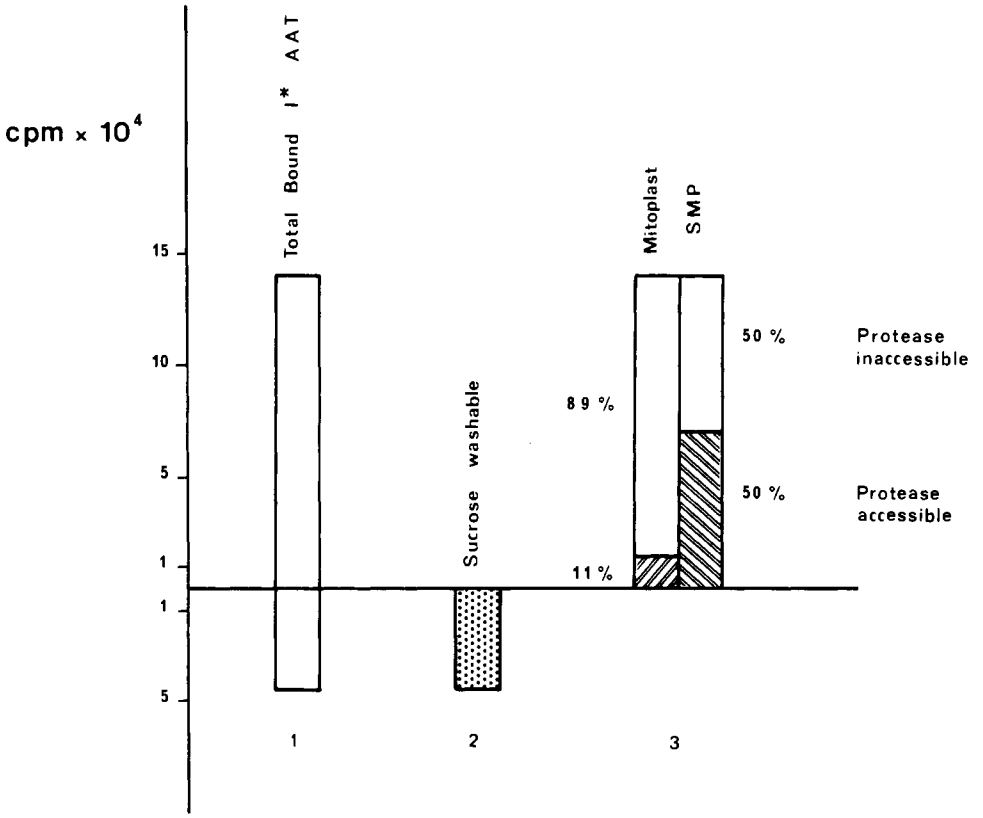


FIGURE 4: Accessibility of reassociated ^{125}I-labeled AAT to mild protease treatment. (1) Total rebound AAT after incubation of submitochondrial fractions with the corresponding fluid (see Materials and Methods). (2) Dotted area: ^{125}I-labeled (I*) AAT released by sucrose washing. (3) Accessibility of ^{125}I-labeled AAT on the two types of submitochondrial fractions: lower hatched area, protease-released fraction; upper white area, protease-inaccessible fraction.

found in the internalized ones is 78%.

This approach considers AAT as an antigen and is in good agreement with previous "enzymatic" experiments. It clearly shows that the enzyme molecules rebind to mitoplasts during internalization incubation and thus negates the possibility of activation.

Labeled Anti-AAT Antibody Binding to Mitoplasts and SMP. A similar molecular approach was used to study the accessibility of rebound AAT from the outside of mitoplasts and SMP when sucrose-incubated and internalized mitoplasts and SMP were incubated with ^{125}I-labeled anti-AAT immunoglobulins. Measurements of the radioactivity thus fixed to the submitochondrial fractions gave the following results: 14 μg of immunoglobulin was bound per mg of protein of sucrose-treated mitoplasts, 14 μg/mg was bound (internalized mitoplasts), and 32 μg/mg was bound (SMP). Results for sucrose-incubated (intact) and internalized mitoplasts are thus very similar. So, with the above precipitation results, it is clear that the rebound enzyme became inaccessible to the antibody during reassociation. This is additional evidence for true internalization. The greater binding to SMP provides a validating control for the method. The ratio of binding to SMP/mitoplast is 2.2:1. Considering the lower specific activity of SMP, as compared to the mitoplast, this ratio becomes 4.4:1 when the results are expressed as micrograms of antibody bound per unit of AAT activity.

Internalization of AAT Measured by the Accessibility of ^{125}I-Labeled Enzyme to Protease. Further molecular evidence was provided by the study of rebound labeled AAT accessibility to protease treatment. Purified mitochondrial AAT labeled with ^{125}I was added to a dialyzed and concentrated fluid prepared from the succinate-treated mitoplast or SMP. The reassociation incubations were performed as done previously, and the accessibility of the labeled enzyme to mild protease treatment was measured as released radioactivity (Figure 4). Iodination led to inactivation of the enzyme activity and rebinding ability. Only 30% of ^{125}I-labeled AAT rebound to submitochondrial fractions. The remaining portion was shown by isoelectrophoresis and autoradiography to be denatured. Of the rebound radioactivity, about 30% was sucrose washable. From the rebound nonwashable radioactivity, 11% was released from mitoplasts by trypsin–chymotrypsin treatment. In contrast, 50% of the rebound radioactivity was released from SMP by such protease treatment, giving further evidence that most AAT which reassociated to mitoplasts was internalized and thus was inaccessible to surface protease digestion.

Internalization of AAT Previously Released into Intermembrane Fluid of Intact Mitochondria. The above results show unambiguously the internalization of AAT into isolated mitoplasts. To demonstrate externalization and internalization of AAT in situ, latency measurements were performed with intact mitochondria. The assays were performed under conditions preserving mitochondrial integrity by taking advantage of the following properties of this organelle: (a) the externalization into the intermembrane space of internally localized protein by the action of a movement effector (Waksman & Rendon, 1974; Waksman et al., 1977); (b) the dependency of this phenomenon upon the fluidity of the membrane and thus upon temperature (Crêmel et al., 1976); (c) the impermeability of the inner mitochondrial membrane to pyridine nucleotides (Lehninger, 1951; Purvis & Lowenstein, 1961).

It has been shown that in the presence of exogenous succinate, malate dehydrogenase, an otherwise latent enzyme, expresses itself in whole mitochondria (Rendon & Packer,

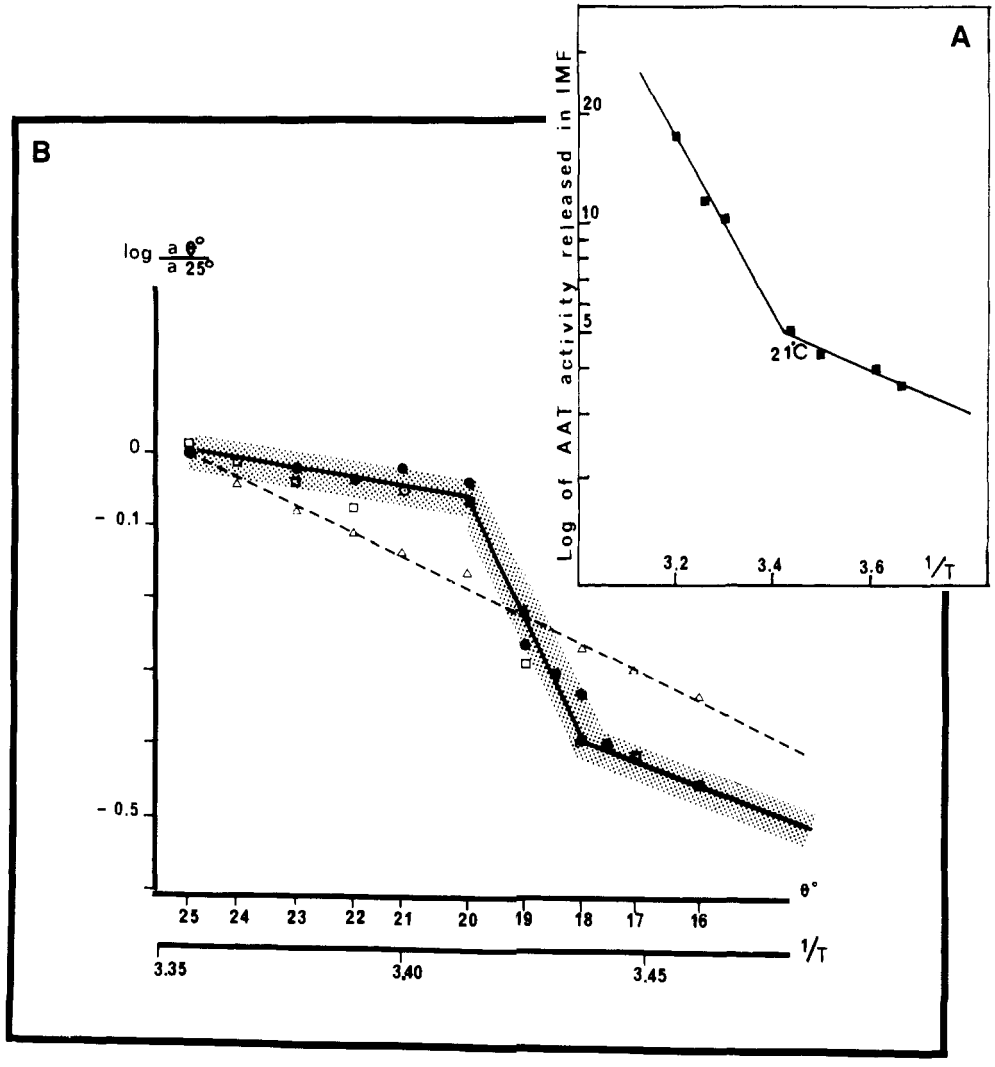


FIGURE 5: Variation of the expression of aspartate aminotransferase (AAT) in intact mitochondria as a function of temperature, in presence of exogenous succinate (1.6 mM). (A) Arrhenius plot of intermembranal AAT release induced by succinate as determined by the digitonin method. (B) AAT activity in intact mitochondria as compared to activity at 25 °C, as a function of 1/temperature. (△) Control: AAT activity in isolated intermembranal fluid as a function of 1/temperature. (□) AAT activity in intact mitochondria as a function of temperature increase (0.5 °C/min). (●) AAT activity in intact mitochondria as a function of temperature decrease (0.2 °C/min).

1976). This was correlated with the intermembrane release of the enzyme (Waksman et al., 1977).

Whole mitochondria incubated under isoosmolar conditions in the presence of exogenously added NADH, malate dehydrogenase, and succinate did not release AAT in intermembrane fluid at a temperature below 20 °C (Figure 5A). When the temperature was slowly increased (0.5 °C/min), a sudden burst of AAT activity appeared between 19 and 21 °C and leveled off rapidly. This phenomenon was reversible upon a slow decrease of temperature (Figure 5B).

The results thus show that AAT became accessible to the exogenous NADH and expressed itself into the intermembrane space following the actions of both succinate and temperature. This accessibility was lost when temperature conditions were reversed, suggesting the reinternalization of the released enzyme.

Conclusions

Previous work has established the existence of intramitochondrial intermembrane protein movements. One of the questions raised by such movements was the possibility of protein translocation across the inner mitochondrial membrane. AAT has been localized by some authors (Scholte et al., 1973; Wit-Peeters et al., 1971) on the inner face of the inner mitochondrial membrane. This was observed either by measuring the latency of the enzyme in whole mitochondria or by studying its Lubrol compartmentation and by measuring its latency in inside-out submitochondrial particles. These measurements were all performed either in 0.25 M sucrose or in low phosphate concentrations, media shown to maintain AAT associated to the mitoplast (Waksman et al., 1977). In the course of our work (Waksman & Rendon, 1974; Waksman et al., 1977), it became clear that this enzyme, as well as other proteins, could change its localization and move to other compartments such as the intermembrane space or matrix upon addition of movement effectors such as succinate to the medium.

After exclusion of technical artifacts (Waksman & Rendon, 1974; Waksman et al., 1977), there remained the possibility of the enzyme passing through the inner membrane during its release. Reversibility of this release would then correspond to internalization of the enzyme. The present report constitutes evidence in favor of the internalization of AAT.

The above results first show that AAT released after succinate addition rebinds to mitoplasts or SMP when succinate is removed from the medium. With dialyzed intermembrane fluid, rebinding of the released enzyme was about 90%, when measured by both the Lubrol technic and the

Table I: Summary of Results

	mitoplast	SMP	
immunoprecipitation	78% (succinate treated, 46%)		total enzyme (intact mitoplast is 100%)
lubrol fractionation	69%		% of reassociated enzyme recovered in the membranal fraction
latency (NADH)	31% (1)	100% (3.2)	nonlatent reassociated enzyme (ratio)
protease treatment	11% (1)	50% (4.6)	accessible reassociated enzyme (ratio)
antibody binding	1	4.4	ratio of binding

latency method. Similar rebinding occurred with SMP. This suggests a highly selective affinity of mitochondrial AAT for the inner mitochondrial membrane. To demonstrate AAT internalization, six experimental approaches have been used here. The first two studied distribution of AAT into mitoplasts, and the remainder investigated the accessibility of rebound AAT to a nonpermeating substrate, to protease treatment, and to a specific antibody.

The results of all these experiments are summarized in Table I. Three methods measured enzymatic activity. These results could be criticized on the basis of AAT activation. Such activation is unlikely since the total activity remained constant, meaning that in order to accept such results, one should admit compensatory inhibition on the complementary compartments under such different assay conditions as sucrose, Lubrol, or buffer media. These results show that an average of about 69% of the total rebound activity became inaccessible from outside the mitoplast.

To further invalidate the possibility of enzyme activation, molecular markers were used. Immunoprecipitation confirmed the similarity of AAT content in sucrose-incubated (control) and internalized mitoplasts as compared with succinate-treated mitoplasts. In the protease method, the enzyme was directly labeled with ^{125}I. The inconvenience of this method was structural modification of about 70% of the protein, as shown by the autoradiography of the isoelectrophoregram. Of the remaining activity, about 70 to 80% reassociated with the mitoplast. Of the rebound activity, only 11% was released from mitoplasts by the trypsin–chymotrypsin treatment, whereas 50% was released from SMP under such conditions. Very similar results were obtained by the antibody binding assays, although the difference of AAT specific activity between mitoplasts and SMP makes these results more difficult to interpret in molecular terms.

Nevertheless, whatever the technics used, the localization of the enzyme in internalized mitoplasts was comparable with that in intact mitoplasts, suggesting that internalization had occurred. With whole intact mitochondria, the observations showed that AAT, an otherwise latent enzyme, expressed itself in the presence of succinate, when the temperature was superior to that of the thermotropic fusion point for succinate-induced intermembrane release of AAT. On decreasing temperature, the enzyme once more became latent. Controls showed that this was not due to the temperature activation of the enzyme. Under these particularly mild conditions (1.6 mM succinate), the increase of activity represented 500% of the activity in the absence of succinate and 30% of the total AAT activity of the mitochondria. Whether during this reversible movement the enzyme was released in situ into the intermembrane space or simply translocated but still bound to the outer surface of the inner membrane remains an open question. In any case, in the presence of succinate, the enzyme was easily released into the medium during fractionation. The present results clearly demonstrate mitochondrial AAT internalization within both the mitoplast and the inner membrane–matrix complex of intact mitochondria. These experiments confirm, at a molecular level, earlier results from our group (Waksman & Rendon, 1971, 1974).

A similar experimental approach has been used by Marra et al. (1978). Their results confirmed part of our previous work (Waksman & Rendon, 1971, 1974) and further indicated the possibility of mitochondrial AAT crossing the outer membrane of intact mitochondria unidirectionally. These authors drew a possibile relationship between AAT movement and lipid composition of the inner membrane. Such a relation had been demonstrated by Crêmel et al. (1976). Likewise, the possibility of ionic strength being the sole factor in this phenomenon has already been examined and rejected.

One of the possible roles of this phenomenon could be related to mitochondrial biogenesis. Aspartate aminotransferase, like most mitochondrial proteins, is synthesized on cytoplasmic ribosomes and transported into the mitochondria. This implies transfer across the mitochondrial membranes (Schatz & Mason, 1974). A mechanism involving larger precursors has been postulated for the transport of these proteins into mitochondria (Maccecchini et al., 1979). With the possible help of conformational changes, the cleavage of the precursor into a mature form could provide the "driving force" for the transfer. This process differs from that described by Kellems et al. (1973) for mitochondrial proteins and by Blobel & Dobberstein (1975) for secretory proteins ("Signal hypothesis") since it does not require vectorial translation of the protein chain during its elongation.

Our experiments demonstrate that, in vitro, transfer of AAT through the inner membrane of mitochondria occurs without vectorial translation and without any modification of a precursor. This had already been suggested in the case of rat liver glutamate dehydrogenase (Godinot & Lardy, 1973) and mitochondrial proteins of *Neurospora crassa* cells (Harmey et al., 1977).

It can be postulated from our findings that the movement effector-induced changes in the conformation and interactions of the membrane and perimembrane proteins and lipids provide the driving force for the observed translocation. Investigations of the molecular mechanisms involved in this phenomenon are currently in progress in our laboratory by the use of chemical modification of the membrane and by the use of physical probes. These procedures should permit detection of structural changes both in membrane and in internalized proteins.

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Localization of the Primary Quinone Binding Site in Reaction Centers from *Rhodopseudomonas sphaeroides* R-26 by Photoaffinity Labeling†

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ABSTRACT: We have prepared a radioactive photoaffinity label, 2-azido[^{3}H]anthraquinone, to substitute for ubiquinone as the primary electron acceptor in reaction centers from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26. When the label was illuminated with ultraviolet light, it photolyzed to yield an intermediate, most likely a triplet nitrene, which was observed at 80 K by optical and EPR spectroscopy. Reacton centers that had the ubiquinone replaced by the label showed photochemical activity (bleaching at 865 nm and light-induced EPR signals) at room temperature, 80 K, and 2.1 K. When reaction centers reconstituted with the label were illuminated with ultraviolet light at 80 K and subsequently warmed, some of the label became covalently attached to the protein. Similar results were obtained with infrared illumination, showing that nitrene formation can be mediated by the tetrapyrrole pigments. Analysis of photolyzed protein samples by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that the M subunit of the reaction center was selectively labeled, as compared to control preparations in which the primary quinone binding site was filled by ubiquinone before labeling. These results show that the primary quinone binding site is located on or very close (within ~5 Å) to the M subunit of the reaction center.

The first steps in bacterial photosynthesis, namely, the absorption of light and formation of a donor–acceptor ion pair, take place in a membrane-bound pigment–protein complex called the reaction center (RC),[1] which has been isolated in a highly pure form from various bacteria. The details of the primary photochemistry and the identities of the molecules involved in the electron transfers are fairly well understood [for recent reviews see Parson & Cogdell (1975), Loach (1977), and Feher & Okamura (1978)]. The most thoroughly

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EPR, electron paramagnetic resonance; RC(s), reaction center(s); LDAO, lauryldimethylamine oxide; AzAQ, 2-azidoanthraquinone; DEAE, diethylaminoethyl; UQ, ubiquinone.

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