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Intramitochondrial Intermembranal Reversible Translocation of Aspartate Aminotransferase and Malate Dehydrogenase through the Inner Mitochondrial Membrane†

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ABSTRACT: The translocation of aspartate aminotransferase, malate dehydrogenase, and bulk protein from the rat liver inner mitochondrial membrane and matrix toward the intermembranal space induced by certain organic acids (movement effectors) has been studied. Experiments involving a two-stage dissolution of the mitochondrial membranes by the use of detergents strongly suggest that enzymes like aspartate aminotransferase can cross the inner mitochondrial membrane providing exogenous movement effector was present. Experiments which measured the changes in membranal distribution of malate dehydrogenase induced by the movement effectors also suggested the occurrence of a similar phenomenon for this enzyme in intact mitochondria. Control experiments revealed that under our experimental conditions, the inner mitochondrial membrane remained impermeable to small molecules, e.g., sucrose, and that the release of aspartate aminotransferease, malate dehydrogenase, insocitrate dehydrogenase, and bulk protein into the intermembranal space in the presence of succinate occurred at a much lower concentration of digitonin than that required to disrupt the inner mitochondrial membrane.

Lateral protein diffusion in biological membranes could be facilitated by the high degree of molecular mobility of the membranal lipids (Gitler, 1972). Thus, the fluid character of a membrane would be the result of the spatio-temporal interactions of its lipid–protein constituents. Changes in these interactions could be caused by modifications to the chemical composition of the membrane itself or by changes in the environment surrounding such a membrane (Branton et al., 1972).

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Structural studies and some functional considerations have suggested that membranes may have a mosaic structure (Singer and Nicolson, 1972). The evidence, however, for the mosaic-type membrane, with a lipid bilayer as framework, is strongest for membranes that are not of the most basic type, i.e. membranes not surrounding a living cell and controlling transport of metabolites in and out of these membranes (Tanford, 1973). Recent studies on protein-rich membranes have led to the formulation of a dynamic conception of the structural organization of the membranes (Pfeiffer et al., 1976; Little and Widnell, 1975; Sandri et al., 1976).

In previous publications (Waksman and Rendon, 1974; Crémel et al., 1976) we have characterized reversible protein movements between the inner mitochondrial membrane plus matrix, and intermembranal space. We showed that the lipid composition of the membrane and the exogenous concentrations of substances such as succinate, oxaloacetate, fumarate, etc.,[1] are parameters involved in this phenomenon. The observed movements were called large amplitude protein movements, to distinguish them from lateral protein diffusion and translocation within the membrane.

These observations raised the possibility as to whether proteins such as AAT[2] could be induced to cross the inner mitochondrial membrane. In the present paper, the translocation of aspartate aminotransferase and malate dehydrogenase from the inner membrane plus matrix compartment toward intermembranal space is demonstrated and discussed.

Materials and Methods

All products used were of the purest quality commercially available. Sodium succinate, oxaloacetic acid, 2-oxoglutaric acid, NADH, and malate dehydrogenase were obtained from Boehringer, Mannheim. Sodium fumarate, citrate, and acetate as well as bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Digitonin was purchased from BDH Chemicals Ltd. Lubrol WX was from Imperial Chemical Industries. ^3H_2O, [U-^{14}C]sucrose, and inuline [^{14}C]carboxylic acid were from Amersham. Monophase (40), Carbosorb, and Permafluor were from Packard Instrument Co., Inc., Downers Grove, Ill.

All the solutions were prepared in 0.25 M sucrose which was also used for the mitochondrial preparations and subfractionations. All centrifugations were performed in a Spinco Model L 50 centrifuge. All manipulations were performed at 4 °C except where otherwise mentioned.

Preparation of Mitochondria and Mitochondrial Fractions. The rat liver mitochondria were prepared according to Harel et al. (1957) as modified by Waksman and Rendon (1974). Prior to separation of submitochondrial fractions, mitochondria were incubated as previously described (Waksman and Rendon, 1974).

For the isolation of submitochondrial fractions, the digitonin method described by Levy et al. (1967) and Schnaitman et al. (1967) as modified by Waksman and Rendon (1974) was used.

For the further separation of the inner matrix from the inner membrane plus matrix complex, the Lubrol technique as described by Chan et al. (1970) was employed. For these experiments, the inner membrane plus matrix complex obtained after digitonin treatment was first incubated for 2 min at 37 °C, either in the presence or absence of 5 mM of the movement effector (this molarity corresponds to the concentration of the effector after digitonin dilution).

Lubrol WX (0.16 mg per mg of protein) was then added and the incubation performed for 15 min at 0 °C. The mixture was centrifuged for 60 min at 40 000 rpm in a 40 type rotor. The pellets were homogenized and resuspended in 2 mL of water.

Enzymatic Assays. Aspartate aminotransferase (AAT) was determined according to Karmen (1955), and for malate dehydrogenaee (MDH) the method of Ochoa et al. (1955) was used. Adenylate kinase (ADK) and isocitrate dehydrogenase (ICDH) were assayed according to Schnaitman and Greenawalt (1968). Protein determination was conducted by the method of Lowry et al. (1951), with bovine serum albumin as standard.

MDH Latency. Mitochondria were prepared in 0.25 M sucrose buffered with 1 mM Tris-EDTA at pH 7.5 and separated as described above. The organelles had a respiratory control greater than 3 in succinate (Na^+).

Latency measurements were performed in a medium containing: 0.1 mg of mitochondrial protein, 0.08 mM oxaloacetate, 0.2 mM NADH, and 1 μg of rotenone in 0.25 M sucrose buffered with 10 mM phosphate (Na^+) at pH 7.0. The final volume was adjusted to 3 mL. Readings were performed in a Cary 16 type spectrophotometer at 25 °C at 340 nm in the presence of increasing concentrations of succinate (Na^+), ranging from 0 to 30 mM. All measurements were started by oxaloacetate addition, after 1.5 min preincubation. After this preincubation no further modifications of readings due to light scattering could be observed.

Measure of Sucrose, Inuline, and Water Spaces. Sucrose and water spaces were measured according to Tarr and Gamble (1966) and Gamble and Garlid (1970). Experiments were performed with increasing concentrations of digitonin in the presence or absence of 20 mM succinate. Incubations were performed in a final volume of 0.5 mL, containing 0.25 μCi of [U-^{14}C]sucrose, 0.25 μCi of ^3H_2O, and 12.5 mg of mitochondrial protein.

A parallel series of experiments was performed replacing the sucrose isotope by 0.25 μCi of inulin [^{14}C]carboxylic acid.

After the addition of sucrose at 0 °C to stop the action of digitonin (3 times initial volume), 0.5-mL aliquots were collected in 1-mL Eppendorf centrifuge tubes and centrifuged for 10 min at 4 °C in a Janetzki type TH 11 table centrifuge.

The pellets were drained and the centrifuge tubes wiped with a tissue. They were recovered in 3 washes of 50 μL of water with the aid of a smooth-edged glass rod and deposited on a slip of Whatman No. 3MM paper. This paper was burned in a Packard 306 Tri-Carb sample oxidizer. $^3H_2$ was collected in 9 mL of Monophase (40) and $^{14}CO_2$ in 6 mL of Carbosorb + 9 mL of Permafluor.

The samples were counted in a liquid scintillation spectrophotometer, Intertechnique Model SL 30.

Results

Influence of Various Movement Effectors on Intramitochondrial Localization of Aspartate Aminotransferase and Bulk Protein. Digitonin treatment separates the inner membrane plus matrix complex from intermembranal fluid and the outer membrane, whereas Lubrol treatment separates the inner

[1] The substances which initiate protein release and binding are referred to as "movement effectors".

[2] Abbreviations used are: AAT, L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1); ADK, ATP:AMP phosphotransferase (EC 2.7.4.3); ICDH, isocitrate dehydrogenase (NADP$^+$) (EC 1.1.1.42); MDH, L-malate:NAD$^+$ oxidoreductase (EC 1.1.1.37); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NAD$^+$ and NADP$^+$, oxidized nicotinamide adenine dinucleotide and NAD$^+$ phosphate, respectively.

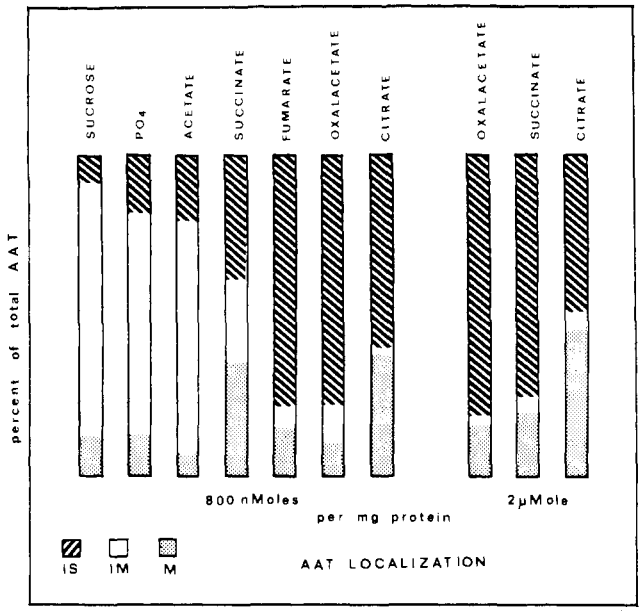

FIGURE 1: Influence of different movement effectors on the intramitochondrial "localization" of AAT. Digitonin treatment (0.8 mg per 10 mg of mitochondrial protein) was followed by Lubrol WX treatment (1.6 mg per 10 mg of mitochondrial protein). Results are expressed as percent of total activity. Effectors were used at either 20 mM (800 nmol per mg of mitochondrial protein) or at 50 mM (2 μM per mg of mitochrondrial protein): IS, intermembranal space; IM, inner membrane; M, matrix.

mitochondrial matrix from the inner membrane.

Figure 1 summarizes the results obtained after these treatments. It is shown that in a 0.25 M sucrose medium, AAT is associated with the inner membrane fraction. However, in the presence of certain of the above-mentioned movement effectors, a totally different distribution of AAT is revealed. With acetate and phosphate, respectively, 71 and 67% of the total AAT is localized in the inner membrane, whereas with the other movement effectors very low AAT activity is recovered in this compartment; with oxaloacetate, fumarate, and citrate only, 5, 10, and 1%, respectively, remain in the inner membrane. Figure 1 shows the distribution of the enzyme between the three compartments, inner membrane, matrix, and intermembranal space as a function of the different effectors used.

To show the reversibility of this, the inner membrane plus matrix fraction was reisolated after movement effector incubation, resuspended in 0.25 M sucrose alone, and treated with Lubrol. In this case, the AAT associated with these two compartments is redistributed (91%) to the isolated inner membrane as in the sucrose control. Movement of AAT from the inner membrane to the matrix and its reversibility from inner membrane to intermembranal space were reported in a previous publication (Waksman and Rendon, 1974).

The change in bulk protein localization follows a pattern comparable to that at AAT except that the phenomenon is less pronounced (Table I).

Malate Dehydrogenase (MDH) Latency as a Function of Increasing Concentrations of Succinate (Na^+). To establish whether or not it would be possible to show protein movement in intact mitochondria, latency of MDH was measured as a function of increasing concentrations of succinate. MDH activity could be detected in intact organelles. This indicates the translocation of MDH activity from the mitoplast to the intermembranal space. This activity curve is parallel to that of the release of MDH into the intermembranal space induced by succinate (Figure 2).

TABLE I: Localization of Bulk Protein in Intermembranal Space (IS), Inner Membrane (IM), and Matrix (M).[a]

Movement effectors	IS	IM	M
Sucrose	23	39	38
Succinate	34	35	31
Oxaloacetate	45	34	21
Fumarate	50	30	20
Phosphate	27	42	31
Citrate	26	33	41
Acetate	31	38	31

[a] Results are expressed as percent of total protein recovered in each subcompartment for 25 mg of initial mitochondrial protein. Movement effectors were used at a concentration of 20 mM.

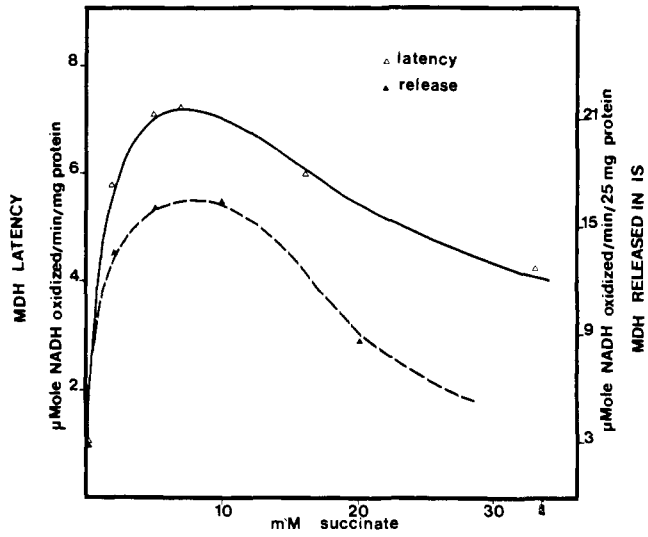

FIGURE 2: Influence of succinate (Na^+) concentration on the malate dehydrogenase latency compared with the release of this enzyme into the intermembranal space. Controls were carried out to show that no activation of MDH occurs in the presence of succinate and that the oxidation of the externally added NADH was due to the MDH activity. In addition, in the presence of succinate, no extramitochondrial release of the enzyme occurs: IS, intermembranal space.

Sucrose, Inuline, and Water Spaces as a Function of Digitonin and Succinate Concentrations. Sucrose and water space were estimated at concentrations of digitonin ranging from 0 to 1.5 mg per 10 mg of mitochondrial protein in the absence or in the presence of 20 mM succinate. Results (Figure 2) show that the sucrose to water ratio: (1) is the same in the presence or absence of succinate; (2) stays at a constant value of 0.8 up to a concentration of 1.25 mg of digitonin per 10 mg of mitochondrial protein (Figure 3A); the value of this ratio is in agreement with Tarr and Gamble (1966) and Gamble and Garlid (1970) when using their data for calculation, and shows, therefore, that the internal membrane remains rather intact up until this concentration of digitonin; (3) the inuline to water ratio increases slightly, and in a parallel fashion, for both sucrose and sucrose plus succinate incubated mitochondria (Figure 3B), suggesting progressive and increasing breakage of external membrane.

Release of AAT, MDH, ICDH, Bulk Protein, and ADK Leakage as a Function of Increasing Digitonin and Succinate Concentrations. The measure of bulk protein and various "marker" enzymes released as a function of digitonin and succinate concentrations led to the following results (Figure 4). (A) ADK, a marker enzyme of the intermembranal space, is totally released in extramitochondrial medium at digitonin

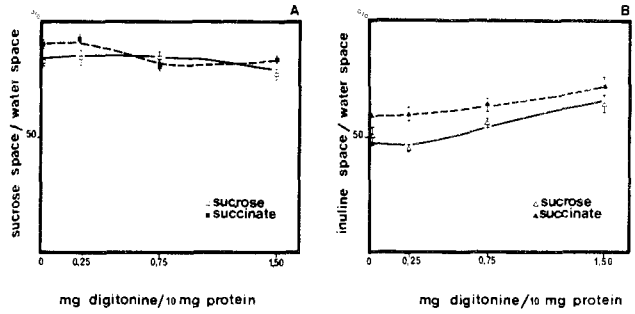

FIGURE 3: Influence of sucrose and sucrose plus succinate (Na^+) on sucrose (A) and "inulin (B) permeabilities as a "function" of digitonin concentration. For the calculation of the spaces the following formula was applied: [^{14}C or ^3H counts in pellet (150 μL of original suspension)/^{14}C or ^3H total counts in 150 μL of original suspension] × 150.

concentrations ranging from 0.5 mg to 1.0 mg per 10 mg of mitochondrial protein. This is independent of succinate concentration, as expected from the localization of this enzyme. (B) For bulk protein and enzyme release, two situations have to be considered. (a) In the absence of succinate, bulk protein, MDH, and ICDH undergo a marked release at digitonin concentrations higher than 1 mg of digitonin per 10 mg of proteins. AAT is not released at all. (b) In the presence of succinate, bulk protein, MDH, ICDH, and AAT are released at digitonin concentrations well below 1 mg of digitonin per 10 mg of mitochondrial protein. The curve showing the enzyme distributions into the intermembranal space is discontinuous (Figure 4), the break above 1 mg of digitonin being due to the disruption of the internal membrane; the break at about 0.4 → 0.5 mg of digitonin is observed only in the presence of movement effector.

Discussion and Conclusion

Our results and data already presented in the literature strongly suggest the possibility of transmembranal protein movements.

In an earlier study, Schnaitman and Greenawalt (1968) showed that AAT was probably associated with the inner matrix complex; using more sophisticated techniques, Scholte (1969) showed that AAT was localized on the inner face of the inner membrane.

We have shown (Waksman and Rendon, 1971, 1974; Rendon and Waskman, 1971) that depending on the external environment AAT could also be localized in the intermembranal space. It thus appears that AAT is able to cross the inner membrane. In the present paper, we first demonstrated that some proteins (i.e. AAT) may be in three different submitochondrial localizations depending upon the nature of the movement effector used. This change in distribution cannot be explained by enzyme activation since total activity in the three compartments measured remains constant. The argument of two separate pools for AAT, one on the outer surface of the inner membrane and one on the inner surface of this membrane, as the sole explanation of these differential localizations has to be rejected. It is difficult to conceive an alternate scheme for the opposite localizations observed with citrate (45% AAT in the intermembranal space) vs. oxaloacetate (82% AAT in intermembranal space) which would fit the present data. Even with an originally asymmetric disposition of AAT on the internal and external faces of the inner membrane it is evident that these observations may only be explained by translocation of AAT from one side of the membrane to the other. Equally these results reveal that this movement may occur in both directions.

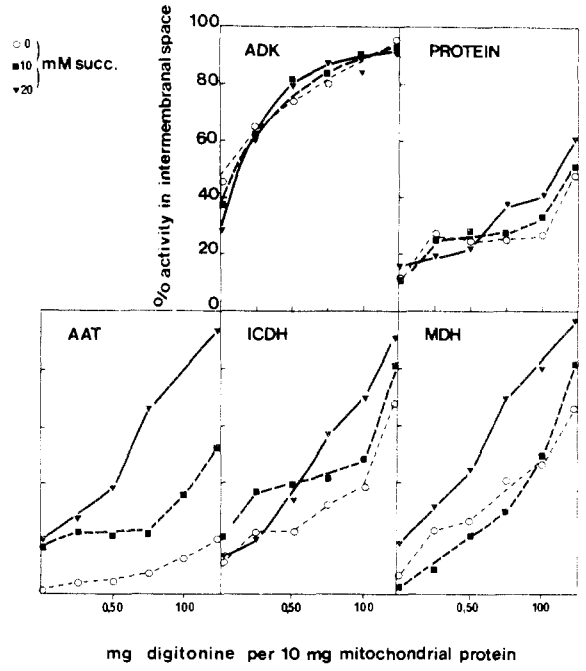

FIGURE 4: Influence of succinate on intermembranal release of ADK, bulk protein, AAT, ICDH, and MDH as a function of digitonin concentration. Enzyme activities and protein concentrations were measured as described under Materials and Methods.

The possibility of technological artefacts was discussed extensively elsewhere (Waksman and Rendon, 1974) and rejected on the following experimental grounds: (1) the release and binding of protein and enzymes occur with mitoplasts or vesicles prepared in three different ways: digitonin, swelling, and shrinking watershocked vesicles; (2) release shows specificity both with regard to the nature of the moving proteins and the movement effectors; (3) the movement is reversible.

However, in the particular case of transmembranal protein movements, a supplementary objection can be raised: does digitonin plus the movement effector "fragilize" in a synergetic manner the inner membrane? In other words, does this treatment create transitory damage which disappears when the movement effector (i.e. physiological mitochondrial substrate) disappears?

Direct experimental evidence against membrane damage was provided by the estimation of sucrose space and the release of "marker" enzymes. (1) It has been shown that the sucrose to water ratio remains constant and parallel for both sucrose and sucrose plus 20 mM succinate-treated mitochondria, and at a digitonin concentration up to 1.25 mg per 10 mg of protein. One has, thus, to exclude the hypothesis of inner membrane damage. Slight, but progressive increases of the inulin to water ratio with increasing digitonin concentrations, in both sucrose and sucrose plus succinate-treated mitochondria, would suggest the gradual breakage of the outer membrane, which would make the intermembranal space now available for inulin. (2) From the release and leakage data obtained after both digitonin and digitonin plus succinate action, it appears that: (a) ADK has an intermembranal localization whether in the presence or absence of succinate; (b) in the absence of succinate, AAT has a rather inaccessible, perhaps tightly bound localization to the inner membrane complex, whereas MDH and ICDH seem to be in a more labile but matricial situation (O'Brien and Matlieb, 1972); (c) in the presence of succinate release of bulk protein, AAT, MDH, and ICDH show a first break at digitonin concentration well below the critical level

of 1 mg of digitonin per 10 mg of mitochondrial protein, corresponding to inner membrane breakage. This suggests release prior to inner membrane damage and, as the sucrose space experiments exclude formation of "holes", one is led to conclude that there is a specific transmembranal translocation of protein, induced by the presence of exogenous succinate.

The fact that, in the presence of succinate ADK, the mobile proteins, AAT, MDH, and ICDH released in the intermembranal space, do not leak out of the mitochondria simultaneously, could perhaps be explained either by differential affinities of the released proteins for the outer constituents of the inner membrane or by the formation of digitonin "holes" in the cholesterol-rich outer membrane. The dimension of these holes would increase with digitonin concentration. Thus, small intermembranal enzymes or molecules such as ADK (mol wt 20 000) or cytochrome *c* (mol wt 13 000) could leak out first, whereas larger molecules such as AAT, MDH, or ICDH (mol wt 10000) could only leave the intermembranal space at a higher digitonin concentration.

A final argument against the hypothesis of inner membrane damage could be found in the latency experiments. In these experiments, performed on intact mitochondria, apparently latent enzymes such as MDH or malic enzymes (Rendon and Packer, 1975; Pfeiffer and Tchen, 1975) only start to express themselves provided the proper specific conditions are used.

In the case of MDH these conditions are precisely those in which this "matrix" enzyme can be localized in the intermembranal space. This strongly suggests that this enzyme moves from the matricial compartment to the intermembranal space.

Thus, experimental and conceptual arguments plead in favor of reversible transmembranal protein movements triggered by the presence of exogenous movement effectors.

How such movements occur in biological membranes and what their physiological consequences are, constitute challenging questions for future study.

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