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# TRANSLOCATION OF MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE THROUGH MITOCHONDRIAL INNER MEMBRANE

## A CROSS-LINKING STUDY WITH DIMETHYLADIPIMIDATE

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## Summary

Mitoplasts isolated from rat liver mitochondria were treated with dimethyladipimidate, a bifunctional alkylating agent. This agent causes, concurrently with modification of amino groups, loss of osmotic response. It was found that after cross-linking, the movement effector, succinate, was unable to induce the aspartate aminotransferase release from mitoplasts. In contrast, dimethyladipimidate-treated mitoplasts were still able to internalize $^{125}$I-labeled aspartate aminotransferase upon removal of exogenous succinate. The possible involvement of membrane asymmetry in the mechanism of translocation of proteins through the inner mitochondrial membrane is discussed.

## Introduction

Earlier studies in our laboratory established that some proteins were able to move reversibly from the mitochondrial inner membrane plus matrix complex to the intermembrane space in rat liver mitochondria [1—3]. These studies, which employed enzymatic and molecular approaches, strongly suggested the internalization of mitochondrial aspartate aminotransferase into the mitoplasts (inner membrane plus matrix) after removal of externalizing agent [4]. As a result, we have become more interested in the relationship between molecular mobility and functional properties of mitochondrial membranes. We were able to establish the relationship between the mobility associated to the lipid moiety of the mitochondrial membrane and the protein translocation

and large amplitude protein movement in general [5,6].

A further approach to investigate the importance of molecular motion in membrane functions is to impose restrictions on motion by artificial means. One way that this can be accomplished is by treating the system with bifunctional alkylating agents. Recent studies have shown that bifunctional imidates inhibit some mitochondrial functions [7—9]. As these reagents caused a loss of osmotic response, it has been suggested that restricted mobility of the cross-linked membrane components might account for the observed effect. The present report describes the effect of cross-linking with the bifunctional imidate, dimethyladipimidate, on the translocation of the mitochondrial aspartate aminotransferase through the inner mitochondrial membrane. Furthermore, the proper effect of amidination was determined using the monofunctional imidate, methylacetimidate.

## Materials and Methods

*Chemicals.* The following were obtained from the sources indicated: methylacetimidate hydrochloride and dimethyladipimidate (Pierce Chemicals, Rockford, IL), Fluram (fluorescamine) (Hoffman-La Roche, Nutley, NJ), Triton X-100 (Sigma Chemical Co., St. Louis, MO) and cetyltrimethylammonium bromide (Serva Feinbiochemical, Heidelberg, F.R.G.). Other reagents were of standard analytical laboratory grade.

Rat liver mitochondria and mitoplasts were prepared in 0.25 M sucrose as previously described [2]. All experiments were performed in fresh preparations.

Stock imidate solutions were prepared as reported [7] in 20 mM Hepes adjusted to pH 8.5 with NaOH. Mitoplasts (1—2 mg protein/ml) were incubated at 0°C with dimethyladipimidate (10 mM) or methylacetimidate (20 mM) in a medium containing 0.25 M sucrose and 10 mM Hepes buffer, pH 8.5. Following treatment, the reaction mixture was diluted 5-fold with ice-cold 10 mM Tris/0.25 M sucrose, pH 7.4, to quench the reaction and the preparations were reisolated by centrifugation. Controls were incubated under identical conditions in the absence of imidates.

For the study of the release (externalization) of aspartate aminotransferase from amidinated mitoplasts, incubations were performed as previously described [1]. Mitochondrial protein (10 mg/ml) was incubated during 5 min at 37°C in the presence of 0.25 M sucrose or sucrose plus different concentrations of sodium succinate. The suspension was then centrifuged for 20 min at 30 000 × *g*. Pellets and supernatants were recovered and assayed for enzymatic activity.

Aspartate aminotransferase reassociation incubations and estimation of the internalized enzyme were carried out as previously described [4]. Mitoplasts freshly isolated from succinate (50 mM)-incubated mitochondria were amidinated as described above. These membranous fractions were then incubated for 5 min at 37°C with an aspartate aminotransferase-enriched intermembranal fluid labeled with $^{125}$I-labeled aspartate aminotransferase. Reassociated mitoplasts were washed with 0.25 M sucrose (sucrose-washable fraction).

Measure of accessibility of $^{125}$I-labeled aspartate aminotransferase in the reasso-

ciated mitoplasts (10 mg mitochondrial protein/ml) was performed by limited protease treatment in the presence of 100 μg of both trypsin and chymotrypsin. After centrifugation (30 000 × *g* for 20 min), samples were counted in an Abott autologic counter type γ counter.

Aspartate aminotransferase activity was determined according to the coupled method of Karmen [10] in the presence of Triton X-100 or cetyltrimethylammonium bromide. Protein were measured by using the method of Lowry et al. [11] as modified by Wang and Smith [12]. Free amino groups were determined fluorimetrically using the fluram reagent [19] in the presence of Triton X-100. To examine the osmotic response of mitoplasts, aliquots were diluted in either 0.25 M sucrose or deionized water and the 620 nm absorbance measured. The ratio ($A_{620\ nm}$ (water)/$A_{620\ nm}$ (sucrose)) was used as an index of osmotic response.

## Results

*Amidination of sucrose- and succinate-incubated mitoplasts*

The imidates apparently react exclusively with primary amino groups [14]. The extent of amidination can be determined by chemical analysis of primary amines. Table I shows the loss of amino groups when mitoplasts were treated for 120 min with comparable reactive concentrations of the bifunctional reagent, dimethyladipimidate, or the corresponding monofunctional reagent, methylacetimidate.

In the case of succinate-preincubated mitoplasts, a similar extent of amidination was obtained both with dimethyladipimidate and methylacetimidate, whereas for the sucrose-incubated mitoplasts, the levels of amidination with dimethyladipimidate were slightly greater than that with methylacetimidate. However, only bifunctional amidination of both sucrose- and succinate-incubated mitoplasts led to an increase in osmotic stabilization; a response which is analogous to that observed with intact mitochondria [9].

TABLE I

EFFECT OF AMIDINATION ON THE OSMOTIC RESPONSE OF SUCROSE- AND SUCCINATE-INCUBATED MITOPLASTS

Amidination of mitoplasts was carried out during 120 min as described in the text. Amino groups were determined fluorometrically with fluram reagent. Aliquots of mitoplast suspension were diluted in 3.0 ml of 0.25 M sucrose or water and the 620 nm absorbance measured. Results are expressed as mean values of six experiments.

| Incubation of mitoplasts | Amidination agent | % free amino groups | Osmotic response ($A_{620\ nm}$ (water)/$A_{620\ nm}$ (sucrose)) |
|---|---|---|---|
| 0.25 M sucrose | none | 100 | 0.31 |
| | dimethyladipimidate | 55 | 1.03 |
| | methylacetimidate | 33 | 0.25 |
| 50 mM succinate | none | 100 | 0.49 |
| | dimethyladipimidate | 60 | 0.82 |
| | methylacetimidate | 54 | 0.47 |

TABLE II

RELEASE OF ASPARTATE AMINOTRANSFERASE FROM AMIDINATED MITOPLASTS

Amidination of mitoplasts was carried out during 120 min as described in the text. Aspartate aminotransferase activity is given in μmol NADH oxidized/min per assay. Amidinated mitoplasts were incubated in 0.25 M sucrose or sucrose plus 2.5 mM succinate. The suspension was then centrifuged and the aspartate aminotransferase activity assayed in the supernatant. Results are expressed as mean values of four experiments.

| Amidination | Aspartate aminotransferase activity | |
|---|---|---|
| | Sucrose | Succinate |
| None | 0.67 | 3.21 |
| Dimethyladipimidate | 1.00 | 0.76 |
| Methylacetimidate | 3.37 | 4.65 |

*Aspartate aminotransferase release (externalization) from amidinated mitoplasts*

Release of aspartate aminotransferase from mitoplasts is induced by movement effectors such as succinate, fumarate or pyruvate. Internalization of the

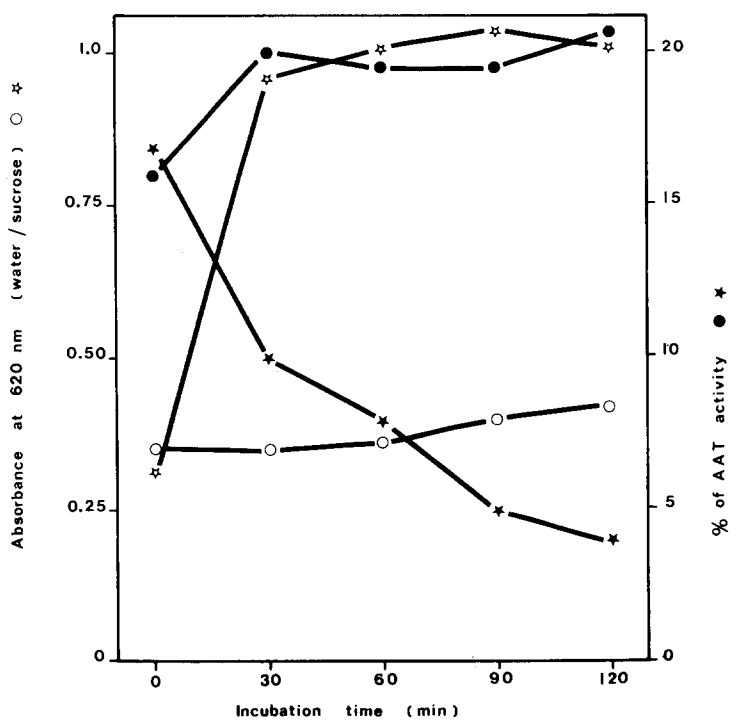


Fig. 1. Time course of the loss in osmotic response by dimethyladipimidate-treated mitoplasts. Effect on the release of aspartate aminotransferase. Mitoplasts were amidinated with 10 mM dimethyladipimidate as described in the text. After reisolating by centrifugation, at the times indicated, mitoplasts were assayed for osmotic stabilization. Release of aspartate aminotransferase was induced by 10 mM succinate. Osmotic response: control (○); dimethyladipimidate-treated (☆). Release is expressed as percent of total aspartate aminotransferase (AAT) activity recovered in the supernatant: control (●); dimethyladipimidate-treated (★).

TABLE III

EFFECT OF AMIDINATION OF MITOPLASTS ON THE ACCESSIBILITY OF REASSOCIATED $^{125}$I-LABELED ASPARTATE AMINOTRANSFERASE TO MILD PROTEASE TREATMENT

Mitoplasts were amidinated as described in the text. Results are expressed in cpm per assay. Figures in parentheses denote percent values with respect to total bound $^{125}$I-labeled aspartate aminotransferase.

| Amidination agent | $^{125}$I-labeled aspartate aminotransferase | | |
|---|---|---|---|
| | Sucrose-washable | Protease-accessible | Protease-inaccessible |
| None | 1650 (23) | 543 (7) | 5080 (70) |
| Dimethyladipimidate | 950 (19) | 377 (7) | 3800 (74) |
| Methylacetimidate | 1100 (27) | 320 (8) | 2660 (65) |

previously released enzyme into the mitoplasts occurs upon removal of the movement effector [2].

Aspartate aminotransferase release induced by succinate was markedly inhibited after dimethyladipimidate treatment (Table II). This effect was not due to the single amidination of amino groups, since aspartate aminotransferase movement is not inhibited after methylacetimidate treatment. Moreover, in the absence of succinate, there is an increase in the enzyme release in methylacetimidate-amidinated mitoplasts when compared to the control. Experiments were carried out to show that there is no activation of the enzyme by methylacetimidate. The time course of the loss of the osmotic response and the inhibition of aspartate aminotransferase externalization were followed when mitoplasts were treated with dimethyladipimidate. The data of Fig. 1 show that aminidation with dimethyladipimidate causes a progressive inhibition of aspartate aminotransferase movement concomitantly with the loss of osmotic response of the mitoplasts.

*Aspartate aminotransferase internalization to amidinated mitoplasts*

The internalization experiments were performed with freshly amidinated, succinate-pretreated mitoplasts, incubated in the presence of intermembranal fluid supplemented with $^{125}$I-labeled aspartate aminotransferase. The accessibility of the reassociated labeled enzyme to mild protease treatment was measured as released radioactivity. Table III shows that mono- and bifunctional-amidinated mitoplasts bound to a lesser extent the $^{125}$I-labeled aspartate aminotransferase when compared to the control. In spite of that, in the three cases about 25% of the total bound radioactivity was sucrose-washable and from the rebound non-washable radioactivity, about 8% was released from the mitoplasts by trypsin/chymotrypsin. Thus, 90% of the aspartate aminotransferase that was not sucrose-washable and that reassociated to the control, methylacetimidate- and dimethyladipimidate-treated mitoplasts, was internalized.

## Discussion

Our results already presented in the literature established the existence of intramitochondrial intermembranal protein movement induced by changes in

the extramitochondrial environment [2]. The in vitro transfer of proteins through the inner membrane of mitochondria raises many questions as to the nature, mechanisms, properties and functions of this phenomenon. In the present study, it has been shown that the use of chemically modified mitochondrial membranes by imidates could bring useful information to this problem. Reaction with bifunctional imidates results in the loss of osmotic response, cross-linking of mitochondrial proteins and resistance to detergent solubilization [7—9]. Thus, the formation of a network has been suggested to account for the observed phenomenon.

It is not possible from our data to establish the nature of the membrane components which were cross-linked in sucrose- or succinate-preincubated mitoplasts. However, our results show that both types of membrane fractions have comparable levels of amidination (55—60% of aminogroups decrease with respect to control).

The results of the present report show that after cross-linking by dimethyladipimidate, the movement effector, succinate, was unable to induce the aspartate aminotransferase release from mitoplasts. On the contrary, cross-linked mitoplasts are still able to internalize the previously released enzyme upon removal of exogenous succinate.

It is known that succinate induces considerable changes in the conformation state of mitochondrial membrane components, including externalization of aspartate aminotransferase and other mitochondrial proteins into the intermembranal space. The reorganization of the membrane prior to the externalization could occur in two steps at least. The first, which takes place between surface charges of the membrane and the movement effector (succinate). The second, a more closed and specific interaction between them. The fact that succinate still interacts with amidinated mitochondrial membranes is illustrated by the presence of an only slightly modified succinate oxidase activity after cross-linking [9].

Furthermore, it is possible to detect, by means of the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid, comparable variations of the fluorescence intensity with both control and dimethyladipimidate-treated mitochondria (unpublished results). Thus, the movement effector is still able to induce conformation changes in mitochondrial membranes without a concomitant release of the enzyme. Since aspartate aminotransferase is located in the inner side of the inner membrane in a sucrose medium [15], it is possible that dimethyladipimidate permeates this barrier and covalently links the enzyme to other membrane components, thus impeding the release event.

Treatment with methylacetimidate does not result in the suppression of succinate-induced release of the aspartate aminotransferase. However, the increase in aspartate aminotransferase levels in amidinated mitoplasts after sucrose or succinate incubation indicates the involvement of primary amino groups in the release of proteins.

Rebinding of released aspartate aminotransferase to inner mitochondrial membranes was observed upon removal by dialysis of the movement effector responsible for release [2]. Furthermore, internalization of the rebound enzyme was demonstrate [4]. In the present investigation, we showed that dimethyladipimidate-treated mitoplasts were still able to internalize $^{125}$I-labeled aspartate aminotransferase.

The formation of a network between membrane components by dimethyladipimidate imposes several restrictions on their mobility. However, on the contrary to the externalization phenomenon, the internalization of the enzyme is not impeded by the partial immobilization of the membrane components. This would suggest that the asymmetry of the membrane together with changes in the membrane environment are an important part of the conditions required for protein translocation through mitochondrial membranes to occur.

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