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PROTON PERMEATION, ADENINE NUCLEOTIDE TRANSLOCATION AND RESPIRATORY CONTROL IN MITOCHONDRIA CROSS-LINKED BY DIMETHYLSUBERIMIDATE

ALVARO RENDON [a,*], RUTH ROTT [b] and YORAM AVI-DOR [b]

[a] *Centre de Neurochimie du CNRS, 11 rue Humann, 67085 Strasbourg Cédex (France)* and [b] *Department of Biology, Technion, Israel Institute of Technology, Haifa (Israel)*

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Summary

Rat liver mitochondria or isolated mitoplasts were treated with the cross-linking agent, dimethylsuberimidate, under conditions (pH 7.5; 0°C) which were not detrimental for the coupling quality of the mitochondria and the effect was evaluated on a kinetic basis. When about 25% of the NH_2-groups reacted, the mitochondria or the mitoplasts acquired complete osmotic stability. Succinate oxidation in state 4 was inhibited by about 30—35%. This effect was also observed when the organelles were amidinated by methylacetimidate, a monofunctional imidate which caused no osmotic stabilization. Uncouplers stimulated succinate oxidation in cross-linked mitochondria to the same extent as in the control, in contrast stimulation by ADP was suppressed. Accordingly, the rate of decay of the respiration-dependent cross-membrane proton gradient was only decreased by 25%, whereas the ATPase and adenine nucleotide translocase were strongly inhibited. In the cross-linked mitochondria, the extent of inhibition of the ATPase and of the translocase was found to be the same whether the assays were performed at 30°C (like the respiratory assay) or at 0°C. The effect of methylacetimidate treatment on these activities at the two temperatures was different. At 30°C, the ATPase was not inhibited and the extent of inhibition of ATP translocation was small. At 0°C, the two activities were nearly as much inhibited as in cross-linked mitochondria. Our results suggest that a considerable rigidity can be introduced in the coupling membrane by cross-linking, without a major loss in the

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* To whom correspondence should be addressed.

Abbreviations: FCCP, carbonylcyanide-*p*-fluoromethoxyphenylhydrazone; Hepes; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

initial step of energy conservation. However, the energy conserved in the proton gradient cannot be utilized for ATP synthesis, probably because of the restricted mobility of adenine nucleotide translocase in the cross-linked mitochondria.

Introduction

Packer and coworkers [1—3] have studied in the past the effect of the bifunctional cross-linking agent, dimethylsuberimidate on rat liver mitochondria with the purpose of assessing whether restriction of the independent movement of membrane components would influence their role played in membrane multienzyme systems. Dimethylsuberimidate-treated mitochondria lose their ability to swell on being transferred from isotonic to hypotonic conditions (osmotic stabilization), show greatly reduced ATPase activity and the different segments of the respiratory chain become inhibited to various extents. Amidination of free amino groups in mitochondria by a monofunctional imidate, ethylacetimidate, to the same extent as with dimethylsuberimidate causes no osmotic stabilization and only little inhibition of the ATPase or the respiratory chain. Since alkaline pH is favourable for the amidination [4], the above authors conduced the reaction at pH 8.5. The high pH, however, is detrimental for preservation of the mitochondria in the tightly coupled state and hence is not well suited for the study of functions connected with energy conservation or transport. In the present work, an attempt was made to find a compromise between the opposing requirements for amidination and preservation of tight coupling in order to study the effect of cross-linking on such delicate functions of the mitochondria as the respiratory control, uncoupler-stimulated ATPase and certain translocation processes. Also the kinetics of osmotic stabilization were studied in relation to the progress of amidination and to the changes which occur in various mitochondrial functions.

Materials and Methods

Chemicals. Special materials were obtained from the following sources: FCCP, fluorescamine, methylacetimidate and dimethylsuberimidate from Pierce Chemical Co., Rockford, IL, U.S.A.; catalase from Calbiochem, Los Angeles, CA, U.S.A.; valinomycin, atractyloside, bovine serum albumin (fraction V), ATP, ADP, rotenone and Hepes from Sigma Chemical Co., St. Louis, MO, U.S.A.; digitonin from Merck, Darmstadt, F.R.G.; [2,8-^{3}H]ATP from New England Nuclear, Boston, MA, U.S.A.

Rat liver mitochondria were prepared as described by Hackenbrock and Hammon [5]. To isolate the mitoplast fraction mitochondria were treated with digitonin [6].

Final suspension of the particles (45 mg protein/ml) was made in a medium containing 4 mM $MgCl_2$, 0.1 mM EDTA, 0.25 M sucrose and 20 mM Hepes buffer which was adjusted to pH 7.5.

Dimethylsuberimidate or methylacetimidate at a concentration of 0.2 and

0.4 M, respectively, were prepared in ice-cold mitochondria suspension medium. The pH of the solution was rapidly adjusted to 7.5 (unless indicated otherwise) with NaOH. Amidination of mitochondria or mitoplasts was performed by mixing 0.9 vol. stock suspension to 0.1 vol. imidate solution, thus bringing the final concentration of dimethylsuberimidate in the reaction mixture to 20 mM and that of methylacetimidate to 40 mM. The amidination was carried out at 0°C and terminated by diluting the reaction mixture sufficiently to prevent any further change in the concentration of free amino groups during the different assays.

The progress of amidination was followed by withdrawing at various time intervals 5-μl samples of the reaction mixture and adding it to 2.5 ml 0.1 M sodium borate (pH 9.0). The concentration of free amino groups was then determined fluorimetrically as described by Udenfriend et al. [7] with fluorescamine. The fluorescence intensity was measured with an Eppendorf photometer equipped with fluorimetric attachment. A 313—366 nm filter was used for the excitation light and a 420—300 nm cut-off filter for the emitted light. To measure osmotic stabilization 10 μl of the imidate-treated or control suspension were added either to 3.0 ml water or to 3.0 ml of 0.25 M sucrose. The light absorbance was determined at 620 nm. The ratio of the absorbance in water to that in sucrose was taken arbitrarily as a measure of osmotic stabilization.

Oxygen consumption was assayed polarographically with an oxygen electrode in a water-jacketed cell with temperature control. The assay was conducted in mitochondria suspension medium also containing 30 mM sodium succinate, 4 μM rotenone, 5 mM inorganic phosphate and mitochondria.

The rate of decay of the proton gradient (cf. Ref. 8) was measured with a glass electrode also inserted in the same cell used for the respiratory measurement. The glass electrode was connected to a Radiometer, model 26-pH meter. The assay medium contained 0.125 M KCl, 0.5 mM Hepes buffer (pH 7.5), 15 mM sodium succinate, 4 μM rotenone, 0.10 μM valinomycin and a sample of the mitochondria. When all oxygen became exhausted by respiration, a proton gradient was established by applying an oxygen pulse (10 units catalase and a small amount of H_2O_2). The collapse of the proton gradient took place either when the oxygen was used up ($O_2 = 0$) or when 0.1 μM FCCP was injected into the system during the aerobic steady state. The respective decay rate constants were calculated from the recorded traces of the decay as described by Karlish and Avron [9].

ATPase activity was determined by measuring the rate of liberation of inorganic phosphate when a sample of the mitochondria was added to the assay medium containing 110 mM KCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.5), 0.1 μM FCCP and 2.5 mM ATP. After 2 min of incubation, the reaction was stopped by the addition of 5% (w/v) of trichloroacetic acid, followed by centrifugation at 10 000 × *g*, for 5 min at 0°C. Inorganic phosphate was determined in the supernatant by the method of Tausky and Shorr [10].

ATP translocation was carried out by the forward exchange reaction essentially as described by Duée and Vignais [11]. The composition of the assay medium was the same as in the ATPase assay. Mitochondria were incubated under the conditions specified in the legend of the figures. The uptake was

started by addition of [2,8-^{3}H]ATP and rapid mixing with the mitochondrial suspension. After a known time, the exchange was stopped by the addition of 25 μM atractyloside, immediately followed by centrifugation in a microcentrifuge (Janetski TH 11). The surface of the mitochondrial pellet was then washed three times with the incubation medium. The incorporated radioactivity was estimated by liquid scintillation counting. Controls were carried out to show that dimethylsuberimidate-treated mitochondria do not interfere with the radioactive estimation.

Protein determination was done by the method of Lowry et al. [12].

Results

The effect of pH on amidination and osmotic stabilization

Rat liver mitochondria were incubated with dimethylsuberimidate at different pH values at 0°C. The low temperature of incubation minimized nonspecific deterioration of the mitochondria. As seen from the results presented in Fig. 1, it is evident that the higher the pH was, the more amino groups were lost and the more complete was the osmotic stabilization. Nonetheless, at pH 7.5, which is already favourable for the preservation of the functional mitochondria, both the amidination and osmotic stabilization occurred at a reasonable rate. Therefore, in the following experiments pH 7.5 was chosen as the standard pH for amidination.

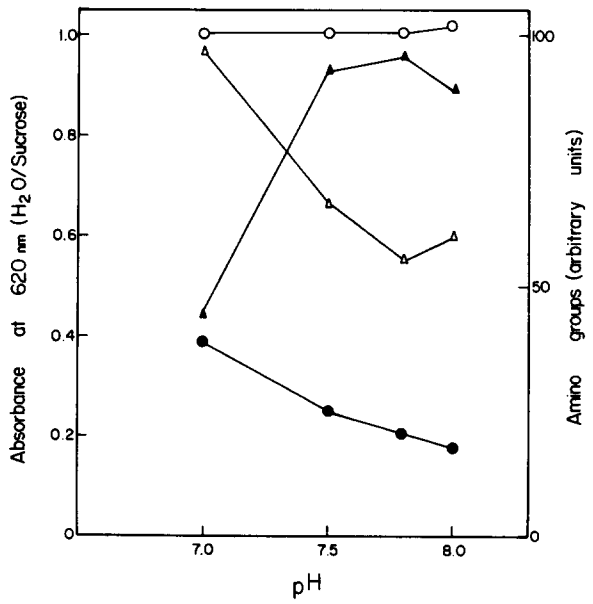


Fig. 1. The effect of pH on the progress of amidination and osmotic stabilization. A stock suspension of mitochondria was incubated, at the pH values indicated, with dimethylsuberimidate at 0°C. The control was exposed to the same treatment in the absence of dimethylsuberimidate. After 180 min of incubation, the concentration of free amino groups (○,△) and the osmotic stability (●,▲) were measured. ○ and ●, Controls; △ and ▲, dimethylsuberimidate treated.

The kinetics of amidination and osmotic stabilization

The monofunctional methylacetimidate and the bifunctional dimethylsuberimidate at equivalent reactive concentrations (40 and 20 mM, respectively) caused the disappearance of free amino groups at similar rates and to similar extents when they were incubated with mitochondria under otherwise identical conditions. However, only in the case of dimethylsuberimidate, osmotic stability was obtained. (see Fig. 2a). Isolated inner membranes (mitoplasts), obtained by digitonin treatment behaved in the above respect like intact mitochondria (Fig. 2b). When about 25% of the NH_2-groups reacted the mitochondria attained full osmotic stability. Repeated additions of dimethylsuberimidate (Fig. 3) increased the extent of amidination without further change in osmotic stability. The curves in Fig. 2a and b, representing the time course of osmotic stabilization, have a sigmoid shape, stabilization being delayed in relation to amidination. It is plausible that first a certain number of cross-links have to be formed before the inner membrane starts to show an increased resistance to swelling.

Effect of amidination on respiration with succinate as the substrate

Mitochondria were incubated either with methylacetimidate or with dimethylsuberimidate until full osmotic stability was attained in the bifunctional reagent containing sample. The respiratory pattern of such amidinated mitochondria is presented in Table I. It is apparent that under state 4 conditions, there was no appreciable difference between the effects of the mono- and bifunctional reagents; i.e. the amidination resulted in 30—35% inhibition of the respiratory rate. When transition from state 4 to state 3 was performed

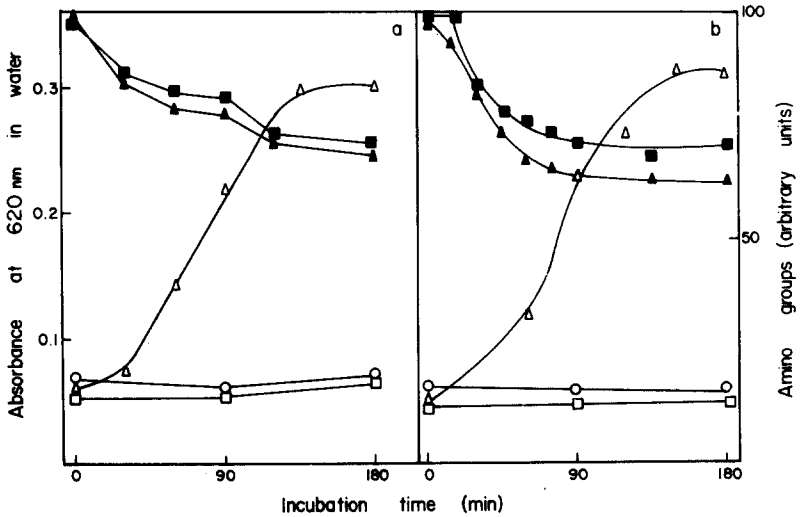


Fig. 2. The time course of amidination of mitochondria (a) and mitoplasts (b) by dimethylsuberimidate and methylacetimidate. Mitochondria or mitoplasts were incubated with both imidates at 0°C at pH 7.5. At the times indicated 5-μl samples were withdrawn for the determination of free amino groups and 10-μl samples to measure the light absorption of the suspension at 620 nm. ○, Controls; △, dimethylsuberimidate treated; □, methylacetimidate treated. Open symbols: absorbance at 620 nm in water; closed symbols: concentration of free amino groups.

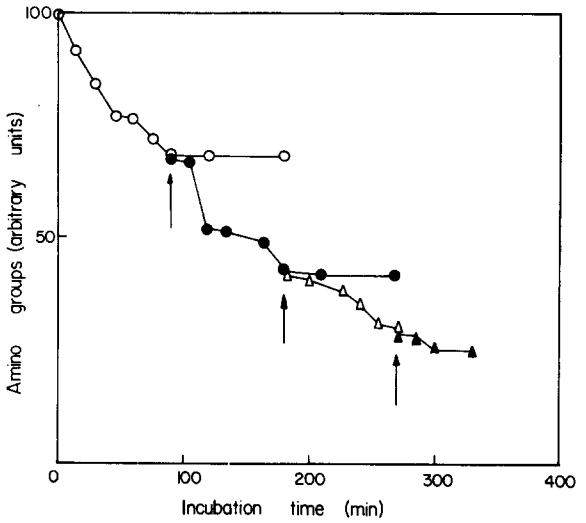


Fig. 3. Effect of repeated additions of dimethylsuberimidate on the extent of amidination. The time course of amidination of mitochondria was followed by determining the concentration of free amino groups at the time intervals indicated. The conditions of the experiments were as described in Fig. 2, except that when indicated by an arrow, dimethylsuberimidate was added again in an amount causing an increment of 20 mM in the concentration of the imidate.

with FCCP, the respiratory control ratio of the amidinated mitochondria was nearly the same as that of the control. In contrast, when state 4 to state 3 transition was induced by ADP, the cross-linking agent caused much stronger inhibition than the monofunctional imidate. Stimulation of respiration by FCCP is known [13,14] to be caused by a collapse of the transmembrane proton gradient, whereas stimulation by ADP requires an adenine nucleotide translocase and a functional ATPase. Therefore, we investigated the effect of the imidates on the rate of decay of the transmembranal proton gradient produced by respiration on the one hand and on adenine nucleotide translocase and the mitochondrial ATPase activity on the other.

TABLE I

THE EFFECT OF AMIDINATION ON SUCCINATE OXIDATION

After incubation at 0°C for 180 min with imidates, samples of 50 μl were withdrawn from the incubation mixture and added to 2.5 ml of the assay medium. The respiratory assay was conducted at 30°C. First, the state 4 respiration was recorded and then 0.1 μM FCCP or 2.5 mM ADP were added as indicated. RCR, rate of respiration before the additions indicated/rate of respiration after the additions.

Pretreatment of the mitochondria	State 4 respiration (natoms O/mg of protein per min)	RCR	
		+FCCP	+ADP
None	50	5.2	2.5
Methylacetimidate	30	5,3	1.9
Dimethylsuberimidate	35	4.6	1.0

TABLE II

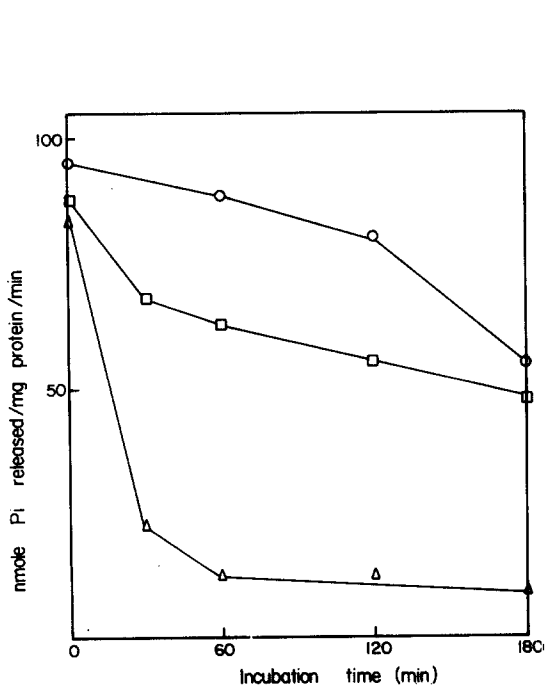
THE EFFECT OF AMIDINATION ON THE RATE OF DECAY OF THE PROTON GRADIENT

After incubation at 0°C for 180 min with the imidate, samples of 0.1—0.2 ml were withdrawn and added to 4.0 ml of the assay medium. DMS, dimethylsuberimidate; MA, methylacetimidate.

Mode of initiation of decay	K_d		
	Control	DMS	MA
$O_2 = 0$	0.34	0.24	0.27
FCCP	0.74	0.86	0.88

Effect of amidination on the rate of decay of transmembranal proton gradient

The rate of decay of the transmembranal proton gradient formed in the respiring mitochondria can be used as a measure for the proton permeability of the inner membrane. The decay takes place when the oxygen becomes exhausted, or can be initiated by adding an uncoupler during the aerobic steady state. Both types of decay were produced in methylacetimidate- and



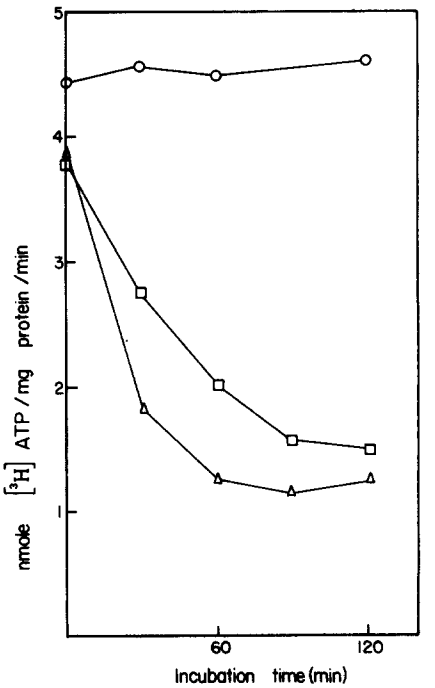


Fig. 4. The effect of amidination on the mitochondrial ATPase activity. At the times indicated 50-μl samples were withdrawn and added to the ATPase assay medium. The amount of inorganic phosphate liberated at 30°C in 2 min was assessed. ○, Controls; △, dimethylsuberimidate treated; □, methylacetimidate treated.

Fig. 5. The effect of amidination on the rate of uptake of [2,8-^{3}H]ATP by mitochondria. At the times indicated samples were withdrawn and added to the adenine nucleotide translocase assay medium. The temperature of assay was 0°C. ○, Controls; △, dimethylsuberimidate treated; □, methylacetimidate treated.

dimethylsuberimidate-treated as well as in control mitochondria. From the recorded traces, the decay rate constants $K_d(O_2 = 0)$ and K_d(FCCP) were calculated. As it can be seen from the data summarized in Table II, both imidates decreased $K_d(O_2 = 0)$ by 20—25%, whereas K_d(FCCP) was not influenced appreaciably.

The effect of amidination on the rate of translocation and of hydrolysis of ATP

The time course of the changes in the activity of the ATPase (Fig. 4) and in the rate of uptake of external ATP (Fig. 5) which occurred when mitochondria were incubated either with dimethylsuberimidate or methylacetimidate was followed. The activity of the ATPase was measured at 30°C as for the other mitochondrial functions described previously but the rate of ATP uptake was assayed at 0°C as described in the literature [11]. The data presented in Figs. 4 and 5 show that incubation of the mitochondria with dimethylsuberimidate caused a progressive inactivation of the ATPase as well as of the adenine nucleotide translocase, reaching its maximum (70—80%) when the mitochondria lost their osmotic sensitivity. In contrast, incubation of the mitochondria with methylacetimidate strongly inhibited ATP uptake without a corresponding suppression of the ATPase activity. Since it is unlikely that external ATP should be split by the mitochondrial ATPase when its exchange with the adenine nucleotides of the matrix is inhibited, we considered the possibility that the difference in assay conditions (30°C vs. 0°C) may be responsible for the apparent inconsistency of the results. To verify this assumption, all the assays were performed at both 0°C and 30°C with the exception of ATP uptake at 30°C in the control mitochondria which was too rapid to be measured with accuracy. Results presented in Table III reveal that in dimethylsuberimidate- as well as in methylacetimidate-treated mitochondria ATP hydrolysis and ATP translocation are of similar order of magnitude, provided that both activities were measured at the same temperature. It is plausible, therefore, that the above generalization also can be extended for the controls when assayed at 30°C for which we do not have experimental data. These results also suggest that after mono- or bifunctional treatment the ATP translocation limits the rate of ATP hydrolysis in the presence of an uncoupler.

Furthermore, from the experiment summarized in Table III, it emerges that

TABLE III

INHIBITION OF [2,8^3-H]ATP UPTAKE AND OF ATPase BY AMIDINATION

After incubation at 0°C for 180 min with imidates, ATPase was assayed as in Fig. 4 and [2,8^3-H]ATP uptake as in Fig. 5, except that the assays were conducted both at 0°C and at 30°C. Data were calculated from initial rates. DMS, dimethylsuberimidate; MA, methylacetimidate.

Assay	nmol/mg protein per min					
	0°C			30°C		
	Control	DMS	MA	Control	DMS	MA
ATP hydrolysis	5.9	0.82	1.15	56	13	52
[2,8^3-H]ATP uptake	4.62	1.24	1.50	—	11.1	32.2

the transition from 0°C to 30°C caused a considerably larger stimulation of the ATPase and of the adenine nucleotide translocase in methylacetimidate-treated mitochondria than in mitochondria treated with dimethylsuberimidate. As a result of this unequal temperature effect the strong inhibition of the two activities observed at 0°C in methylacetimidate-treated mitochondria were partly or entirely relieved when the ATPase and adenine nucleotide translocase were assayed at 30°C.

Discussion

The data of the present work indicate that osmotic stabilization of the mitochondria is the result of the interaction of the cross-linking agent with a relatively small fraction of the available amino groups which are probably located mainly on the inner membrane. The formation of a network could impose severe restrictions on volume changes whilst leaving some essential functions of the coupling membrane largely intact.

Indeed, in the osmotically stabilized mitochondria, the rate of electron flow between succinate and oxygen or the extent of stimulation of succinate oxidation induced by uncouplers was the same as after methylacetimidate treatment and only lower by 35% than in the control. The coupling membrane retained its impermeability to protons since it was possible to generate a proton gradient by oxygen pulse which decayed at a similar rate than in the control preparation.

The inhibition of the adenine nucleotide translocase seems to be directly responsible for both the observed inhibition of the ATPase and for the suppression of ADP-dependent release of the respiratory control in dimethylsuberimidate-treated mitochondria. This is indicated by the finding that under all conditions tested the rate of ATP hydrolysis was similar to that of the uptake of external ATP. One cannot, however, exclude the possibility that dimethylsuberimidate permeated the inner membrane and had a direct effect on the ATPase, since cross-linkers are known [15,16] to produce covalent links between certain subunits of soluble F_1-ATPase accompanied by inactivation of the enzyme.

It is noteworthy that raising the assay temperature from 0°C to 30°C caused a nearly 50-fold increase in the ATPase activity and 30-fold stimulation of the activity of the adenine nucleotide translocase in methylacetimidate-treated mitochondria compared to 15- and 10-fold stimulation, respectively, after dimethylsuberimidate treatment. According to Hackenbrock and coworkers [17,18] below the temperature of 9°C phase separation starts in mitochondria and integral proteins like the translocase tend to be forced out from the gel-liquid phase and to be immobilized. Reduced mobility is likely to impair the function of the translocase. It is tempting to speculate that amidination of membranal lipids and/or the translocase protein might facilitate its extrusion from the gel-liquid phase at temperatures below 6°C, the break point in the Arrhenius plot of the translocase [19]. At 30°C, then, the effect of amidination will not be expressed. In contrast, dimethylsuberimidate treatment may affect the mobility of the translocase also by forming molecular

aggregates through cross-linking. This latter type of immobilization cannot be reversed by raising the assay temperature to 30°C.

Acknowledgements

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