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Localization of the ATP/ADP translocator in the inner membrane and regulation of contact sites between mitochondrial envelope membranes by ADP.
A study on freeze-fractured isolated liver mitochondria

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The frequency of contacts between the mitochondrial envelope membranes was determined in freeze-fractured samples of isolated mitochondria by means of quantifying the frequency of fracture plane deflections between the two membranes. It was observed that the formation of contacts correlated with the concentration of free ADP despite of inhibition of electron transport by antimycin A. The activity of ATPase partially inhibited by oligomycin or depletion of membrane potential by K^+ and valinomycin had no effect on the induction of the contacts by ADP. ATP was ineffective in creating contacts irrespective of the presence or absence of a membrane potential, whereas carboxyatractyloside induced the contacts under all conditions in a manner similar to ADP. These results suggest the involvement of the ATP/ADP translocator in regulation of contact sites. As a consequence, we analyzed its distribution in the inner membrane of kidney and liver mitochondria by binding of [^{3}H]atractyloside to subfractions of this membrane. The experiments demonstrated that the translocator was located in the peripheral part of the inner membrane as well as in the portion which formed the cristae.

Introduction

Contacts between the two envelope membranes have been described in thin-sectioned [1] and freeze-fractured [2] mitochondria as well as in chloroplasts [3]. Such contacts are important for the uptake of newly synthesized mitochondrial proteins [4,5] and for regulation of metabolite exchange [6].

It has been assumed that the contact sites are responsible for the deflections of the fracture plane between the two mitochondrial envelope membranes seen in freeze-fractured mitochondria [2,7]. Quantification of the fracture plane deflections demonstrated that contacts increase in isolated, phosphorylating (condensed, with separated envelope membranes) mitochondria, whereas their frequency is low in state 4 (orthodox, with closely aligned envelope membranes). This suggested that the formation of contacts was not related to the metabolically linked structural (orthodox/condensed) transformation described by Hackenbrock [8,9].

In intact cells, where mitochondria are in state 3, the frequency of contacts changed in different metabolic situations. Hormones like glucagon [10] as well as metabolites such as fatty acids [11] decreased the contacts. In highly glycolytic tumor cells the mitochondria appear to be void of contact sites although these mitochondria were found to be well coupled as they were capable of producing 50% of the cellular ATP [12].

In view of these findings, we attempted to identify a mitochondrial regulatory mechanism that controlled the frequency of contact sites. It was known from earlier investigations [7] that uncoupling by DNP and decoupling by free fatty acids reduced the contacts, suggesting that the contact formation was not regulated by the activity of electron transport. However, using electron microscopy [13] and analysis of the isolated contact sites we observed that a number of kinases are organized in these sites [14]. This indicated that contact formation may induce a specific kinase topology which is required for optimum ADP supply to the adenylate translocator located at the surface of the inner membrane. To elucidate the mechanism of contact formation, we analyzed the frequency of contacts while controlling the rate of mitochondrial ATP synthesis by (i) inhibition of elec-

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tron transport, (ii) varying the free ADP concentration, (iii) inhibition of the adenylate translocator with carboxyatractyloside, and (iv) graded blocking of the ATPase with oligomycin.

Materials

Rats, strain Chbb : THOM (200–300 g body weight) fed with a standard diet, were used for all experiments. All reagents were obtained from Boehringer-Mannheim or Merck, Darmstadt, F.R.G.

Methods

Preparation of mitochondria from rat liver

Livers from rats (250 g body weight) were used for isolation of mitochondria by differential centrifugation in 0.25 mM sucrose buffered with 10 mM Hepes (pH 7.4). The mitochondrial sediment was washed two times using 6000 × g and 3000 × g (Sorvall, rotor SS-34) for sedimentation.

Incubation with [^{3}H]atractyloside

Outer and inner membrane and contact sites were prepared as described recently [14]. 1 mg of each mitochondrial subfractions was incubated for 10 min at room temperature with 11 nmol of [^{3}H]atractyloside. The membranes were washed with sucrose medium by centrifugation and the bound radioactivity was determined in a liquid scintillation counter.

Mitochondrial samples for freeze fracture

The concentration of mitochondria in the samples used for freeze fracture was 10–15 mg per ml. To avoid anaerobiosis, these samples were gassed with carbogene (95% O_2, 5% CO_2). Under these conditions, the diffusion rate of O_2 per ml of oxygen free sample was 860 nmol O_2/min. This was comparable to the maximal O_2 consumption of a mitochondrial suspension (with 20 mg/ml of protein) of 900 nmol/min per ml. It was determined that the level of substrates in the concentrated mitochondrial suspension, used for freeze fracture, was sufficient to allow at least 1 min of maximal state 3 oxidation. The activity of the oxidative phosphorylation, the degree of coupling, and the oxidation rates were monitored by oxygen electrode after 10-times dilution of the mitochondrial suspension.

Respiration measurements

The respiration was determined with a Clark-type oxygen electrode at 22° C according to Estabroock [15].

Freeze-fracture analysis

The activity of oxidative phosphorylation in isolated mitochondria was adjusted by monitoring the oxygen consumption. Aliquots of the mitochondrial suspensions were directly subjected to rapid freezing by the sandwich technique as described by Knoll et al. [16]. Time to cryofixation was approx. 15 s. The samples were broken in a Balzers BAF 301 freeze-etch device at -100°C and $(2–7) \cdot 10^{-7}$ Torr, followed by Pt/C and C shadowing. A Hitachi H7000 instrument at 75 kV was used for electron microscopy.

The morphological evaluations were performed using a CRP graphic board connected to an IBM-compatible AT computer. The nomenclature of the exposed membranes follows that of Branton et al. [17].

As a means of quantifying the difference in fracture-plane deflections, the length of the edge where the fracture plane deflects was measured as it related to the corresponding mitochondrial area. In convex fractures, the length of the edge of the exoplasmic face of the outer membrane was measured, whereas, in concave fractures, the deflection line on the exoplasmic face of the inner membrane was determined. These measurements of L were expressed as length (μm) per unit of mitochondrial fractured membrane area.

In every population of mitochondria there are some which are completely void of fracture plane deflections. The number being dependent upon the metabolic state of the whole sample. To compensate for these differences we adjusted our calculation of fracture plane deflections by first determining on survey pictures the total area of mitochondria with no deflections, M_s, and those with deflections, M_p, and then normalizing these values by the expression $M_p/(M_p + M_s)$. The final value for quantification of freeze-fracture deflections L_p was then calculated from the equation:

$$L_p\ (\mu m/\mu m^2) = LM_p/(M_s + M_p)$$

and the statistical differences obtained by applying the U-test.

The measurements were made in the areas where the curvature was low to avoid large distortions of the measured edge lines. Since the length L_p was based on the specific area of the mitochondrial surface, it was important to establish a method for standardization of the actual area which was used to determine L. As depicted in Fig 1, two different ways to define the area were compared. First, the area was chosen according to the intensity of shadowing, meaning that those parts where the curvature was high and the shadowing was either strong or virtually absent were excluded. Secondly a circular area was constructed inside each mitochondrion using a center point at the intersection of length and cross diameter. The radius of the circle used was 70% of the mean of the length and the cross diameter.

Mitochondria in state 4 from two separate experiments were analyzed using both techniques. There was no significant difference between the two methods (Table I) whether the two samples were analyzed with the same method or the same sample was evaluated with both methods. In the present experiments the area was defined by the intensity of shadowing.

Results

Influence of electron transport activity on formation of contact sites

A thick mitochondrial suspension of 10–20 mg protein per ml was used for freeze fracture. We examined that the substrate concentrations used in the experiments were sufficient to allow maximal state 3 respiration for at least 1 min. In order to compare our data with Hackenbrock's orthodox/condensed transformation [8,9] we used his slightly hypotonic (0.113 M) medium in all experiments.

It is known from earlier investigations [7] that uncoupling by DNP and decoupling by free fatty acids [11] reduce the contacts, suggesting that the contact formation was not regulated by high activity of electron transport. On the other hand the electron transport could also be inhibited without having an effect. When the free ADP concentration was 10 mM, we observed a high frequency of contact sites irrespective of the complete inhibition of electron transport by antimycine A. In contrast, the frequency of fracture plane deflections was significantly lower in mitochondria in state 4 (minus ADP) either in the presence or absence of antimycin A (Table II). This is also evident from the lower number of patches seen in the electron micrographs covering the protoplasmic face of the outer membrane (Fig.2A) in state 4 mitochondria compared to the high number of patches remaining on the surface of the inner membrane in state 3 in the presence of antimycin A (Fig 2B). Although these differences in fracture plane deflections were not always statistically significant when de-

TABLE I

Comparison of two different methods of evaluation

Freeze fractures of two different samples of mitochondria in state 4 were analyzed and the length ($L1$ and $L2$) of the edge where the fracture plane deflects was determined either in an area which was defined by the intensity of shadowing (A) or by the construction of a central circle (B) as shown in Fig. 1. P shows the level of the significance test for the difference between the two methods and P^* shows the significance level for the analysis in two different groups ($L1$ and $L2$) with the same method.

	Applied method	
	A	B
$L1$ (μm^{-1})	9.21 ± 3.96	7.04 ± 3.03 $P \leqslant 0.05$
$L2$ (μm^{-1})	7.82 ± 5.18	8.13 ± 4.57 P (n.s.)
	$P^* < 0.05$	P^* (n.s)

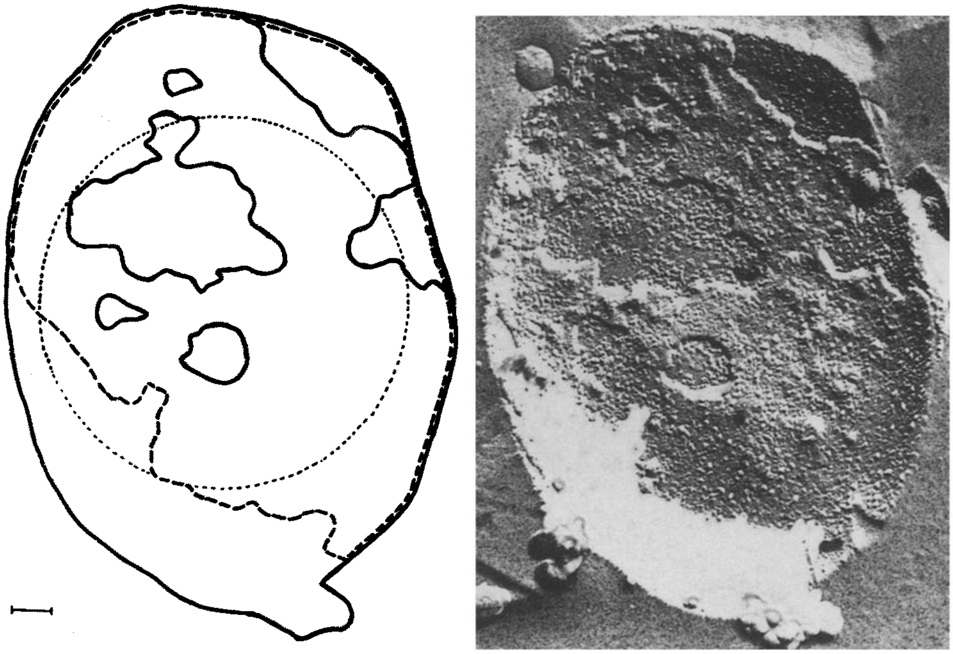

Fig. 1. Method to determine the frequency of fracture plane deflections. As a means of quantifying the difference in fracture plane deflections, the length of the edge where the fracture plane deflects was measured as it related to the corresponding mitochondrial area. The values L were expressed as length (μm) per unit of mitochondrial fractured membrane area. The quantification was made in areas where the curvature was low to avoid large distortions of the measured edge lines. Two different ways were used to define the area: firstly the area was chosen according to the intensity of shadowing (------). Secondly, a circular area was constructed inside each mitochondrion using a center point at the intersection of the length and cross diameter (······). The radius of the circle used was calculated from 70% of the mean of the length and cross diameter. Bar = 0.1 μm.

TABLE II

Effect of inhibition of electron transport by antimycin A on the frequency of contacts

Mitochondria (12.5 mg/ml) were incubated in 113 mM sucrose, 10 mM K_2HPO_4 (pH 7.4), 5 mM $MgCl_2$, 12.5 mM succinate. The stimulation of the oxidation rate by 10 mM ADP was inhibited by addition of 30 ng/mg antimycin A. In a second sample mitochondria were treated with antimycin in the same way but in the absence of ADP. The oxidation rate was determined in the same assays but with a 10-times lower concentration of mitochondria. Maximal state 3 respiration with 10 mM ADP was 68 nmol O_2 min^{-1} mg^{-1}. M_p is the percentage of mitochondria with fracture plane deflections in the whole sample, L represents the mean length in single mitochondria of the edge where the fracture plane deflects. L_p gives the L value corrected for the whole mitochondrial population as described in Methods. The significance of the difference between each group was tested by the U-test with $P < 0.01$.

	State 4	State 3	State 4 + antim.	State 3 + antim.
Oxidation rate in % of maximal state 3 oxidation	11.7	100	2.9	10.3
ADP consumption by oxidative phosphorylation (nmol/min × 2.3 mg)	0.0	552.0	0.0	0.0
M_p (%)	33.9	77.2	42.1	64.4
L (μm^{-1})	5.00 ± 2.01	5.76 ± 2.00 (n.s.)	4.85 ± 1.24 (n.s.) $P \leqslant 0.05$	6.18 ± 1.67 $P \leqslant 0.05$ (n.s.) $P \leqslant 0.01$
L_p (μm^{-1})	1.69 ± 0.68	44.4 ± 1.54 $P \leqslant 0.01$	2.03 ± 0.52 $P \leqslant 0.05$ $P \leqslant 0.01$	3.97 ± 1.07 $P \leqslant 0.01$ (n.s.) $P \leqslant 0.01$

termined as L in individual mitochondria, the percentage, M_p% of patchy mitochondrial fracture planes in the whole state 4 population was always much lower, resulting in a lower L_p value for the whole chondriom.

TABLE III

Frequency of fracture plane deflections correlated to the concentration of free ADP

Mitochondria (20.5 mg/ml) were incubated with different activity of yeast hexokinase in 113 mM sucrose, 10 mM K_2HPO_4 (pH 7.4), 5 mM $MgCl_2$, 12.5 mM succinate, 10 mM glucose and 5 mM ATP. The oxidation rate was determined in the same assay but with a 10 times lower concentration of mitochondria. State 4 respiration was 7.7 nmol O_2 min^{-1} mg^{-1}, the maximal state 3 respiration with 10 mM ADP was 44,1 nmol O_2 min mg. Abbreviations and statistical evaluation as in Table I.

Activity of hexokinase added (mU):	0.0	147	338
Oxidation rate in % of maximal state 3 oxidation	27.6	52.5	85.7
ADP consumption by oxidative phosphorylation (nmol/min × 2 mg)	35.8	123.5	240.6
M_p (%)	52.02	80.14	75.80
L (μm^{-1})	5.55 ± 1.96	5.31 ± 2.56 (n.s.)	12.21 ± 7.89 $P \leqslant 0.01$ $P \leqslant 0.01$
L_p (μm^{-1})	2.89	4.26 $P \leqslant 0.01$	9.25 $P \leqslant 0.01$ $P \leqslant 0.01$

Furthermore, the L and M_p% values changed in the same relation depending on the different states, in spite of the fact that the absolute values varied. This appeared to relate to the batch of mitochondria and was not correlated with the used experimental conditions.

Regulation of contact sites by free ADP

In a second set of experiments we varied the free ADP concentration in the samples by addition of different amounts of yeast hexokinase in the presence of glucose and ATP (Table III). The different rates of state 3 respiration were adjusted by adding hexokinase to the concentrated mitochondrial suspension which was used for freeze fractures. However, the activity of oxidative phosphorylation was determined more precisely after 10 times dilution of the suspension. Considering the actual P/O ratio, the activity of oxidative phosphorylation was calculated from the difference between the oxidation rates in state 4 and that following initiation of the reaction with ATP. According to this calculation, the activity of the oxidative phosphorylation was 27% of the maximal state 3 activity (at 10 mM ADP) when 5 mM ATP was added in the absence of any hexokinase activity. This residual activity results from ADP production by ATPase in damaged mitochondria. Upon addition of hexokinase, the activity of oxidative phosphorylation gradually increased to either 53% or 86% of the maximal state 3 activity (Table III). Under these conditions, the fracture plane deflections in the freeze-fractured samples increased in correlation to the activity of the oxidative phosphorylation.

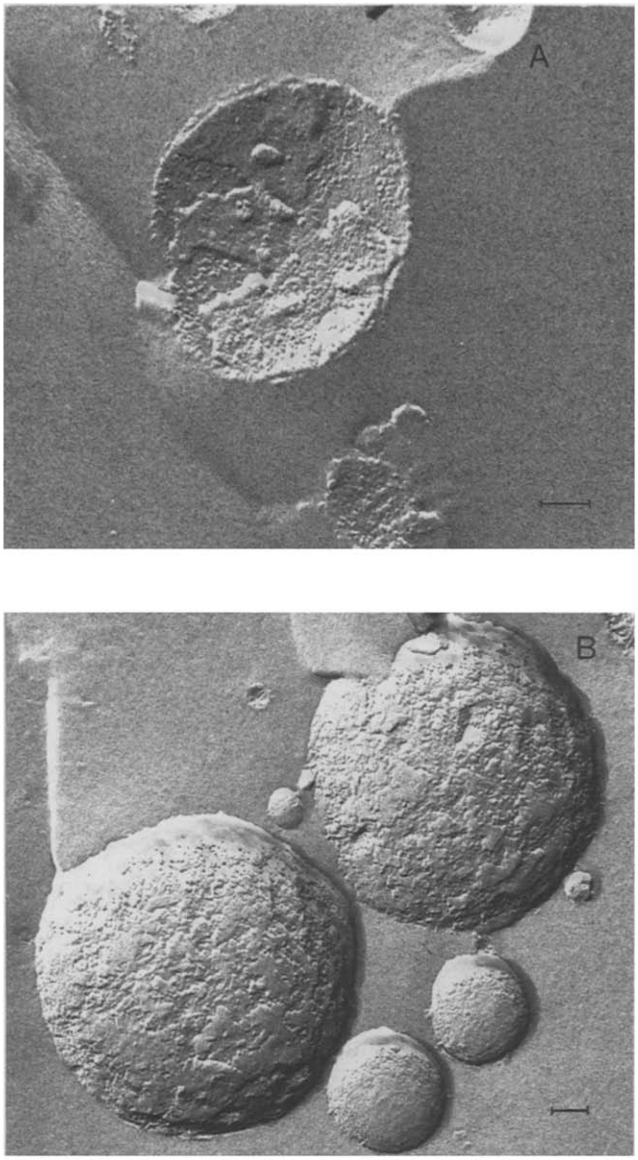

Fig. 2. Freeze fracture of mitochondria in different functional states. Liver mitochondria were incubated in a medium containing 113 mM sucrose, 10 mM K_2HPO_4 (pH 7.4), 5 mM $MgCl_2$, 12.5 mM succinate. (A) State 4 ADP was lacking. (B) 5 mM ADP was added during complete inhibition of the electron transport by the presence of 57.1 ng/mg of antimycin A. Bar = 0.1 μm.

When the frequency of fracture plane deflections (L) was determined in single mitochondria there was no significant difference between mitochondria respiring at 27% of state 3 activity compared to those with 53% of maximal activity of oxidative phosphorylation. However, the percentage of mitochondria exhibiting fracture plane deflections in the former population was significantly lower. When this observation was taken into account, the corrected $L_p$value was significantly higher in the two more actively respiring samples indicating that the total number of contacts in the whole chondriom gradually increased proportional to the activity of the oxidative phosphorylation. Furthermore, these data showed that 5 mM ATP (first sample Table III) was not able to induce the contact sites.

Effect of varying activity of oxidative phosphorylation at constant ADP

The experiments described above suggested that the concentration of free ADP at the mitochondrial surface might be responsible for the regulation of contact sites. However, since the presence of ADP also regulates the activity of oxidative phosphorylation the latter had to be considered as a possible regulatory mechanism. To answer this question, we reduced the activity of oxidative phosphorylation by limitation of phosphate in the presence of high concentrations of ADP (Table IV). Although the activity of oxidative phosphorylation in the absence of phosphate was as low as in state 4 a high frequency of contacts was observed supporting the hypothesis that the high concentration of ADP present in this sample was responsible for this induction.

Effect of inhibition of ATPase on the frequency of contacts

In order to provide further evidence for the importance of ADP in contact formation, we inhibited the activity of mitochondrial ATPase by oligomycin. The samples were subjected to rapid freezing in conditions where the activity of oxidative phosphorylation was reduced by 77% or 82% of the maximal respiration in

TABLE IV

Regulation of oxidative phosphorylation at constant ADP by limitation of phosphate

Mitochondria (17.2 mg/ml) were incubated with either 10 mM phosphate or ADP in 113 mM sucrose, 5 mM $MgCl_2$, and 12.5 mM succinate. The oxidation rate was determined in the same assay but with a 10-times lower concentration of mitochondria. State 4 respiration was 11.2 nmol O_2 min^{-1} mg^{-1}, maximal state 3 respiration with 10 mM ADP was 55.9 nmol O_2 min^{-1} mg^{-1}. Abbreviations as in Table I.

	State 4 ADP lacking	State 4 P_i lacking	State 3
Oxidation rate ni % of maximal state 3 oxidation	20.0	20.0	100
ADP consumption by oxidative phosphorylation (nmol/min × 2.3 mg)	0.0	0.0	411.2
M_p (%)	26.39	50.25	58.48
L (μm^{-1})	5.68 ± 2.35	7.19 ± 4.42 (n.s.)	7.24 ± 3.91 $P \leqslant 0.01$ (n.s.)
P_p (μm^{-1})	1.50 ± 0.67	3.61 ± 2.22 $P \leqslant 0.01$	4.24 ± 2.29 $P \leqslant 0.01$ (n.s.)

TABLE V

Effect of inhibition of oxidative phosphorylation by oligomycin on the frequency of contacts

Mitochondria (14.0 mg/ml) were incubated with different concentrations of oligomycin in 113 mM sucrose, 10 mM K_2HPO_4 (pH 7.4), 5 mM $MgCl_2$, 12.5 mM succinate, and 10 mM ADP. The oxidation rate was determined in the same assay but with a 10-times lower concentration of mitochondria. State 4 respiration was 3.25 nmol O_2 min^{-1} mg^{-1}, maximal state 3 respiration with 10 mM ADP was 42.3 nmol O_2 min^{-1} mg^{-1}. Abbreviations as in Table I.

	State 4	State 3	Oligomycin (ng/mg)	
			260	130
Oxidation rate in % of maximal state 3 oxidation	7.6	100	19.2	23.1
ADP consumption by oxidative phosphorlation (nmol/min × 2.3 mg)	0.0	359.3	44.9	59.9
M_p (%)	48.23	75.95	83.23	80.34
L (μm^{-1})	9.21 ± 3.96	12.77 ± 4.42 $P \leqslant 0.01$	10.42 ± 5.54 (n.s.) $P \leqslant 0.05$	8.98 ± 4.66 (n.s.) $P \leqslant 0.01$ (n.s.)
L_p (μm^{-1})	4.44	9.70 $P \leqslant 0.01$	8.66 $P \leqslant 0.01$ (n.s.)	7.21 $P \leqslant 0.01$ $P \leqslant 0.01$ (n.s.)

state 3 (Table V). As described above, the L and the L_p value of state 4 and state 3 differed significantly in this experiment. However, inhibition of the ATPase by oligomycin resulted in a frequency of fracture plane deflections comparable to those under state 3 conditions. The results demonstrated that the contacts were induced by ADP in spite of the fact that the ATP production by the ATPase was almost completely suppressed.

Effect of inhibition of ATP/ADP exchange on contact site formation

In the light of our observations that the free ADP at the surface of the inner membrane was regulating the contact formation we investigated the possibility that the adenylate translocator was involved in this effect. Upon saturation with ADP, the translocator would be orientated towards the inner surface of the inner membrane. The binding of ADP can be suppressed by

TABLE VI

Effect of carboxyatractyloside on contact formation

Mitochondria (12.5 mg/ml) were incubated in 113 mM sucrose, 10 mM K_2HPO_4 (pH 7.4), 5 mM $MgCl_2$, 12.5 mM succinate. The stimulation of the oxidation rate by 10 mM ADP was reduced by addition of 0.8 nmol carboxyatactyloside (C-atract.) per mg. In a second sample mitochondria were treated with carboxyatractyloside in the same way but in the absence of ADP. The oxidation rate was determined in the same assay with a 10-times lower concentration of mitochondria. Maximal state 3 respiration with 10 mM ADP was 68.0 nmol O_2 min^{-1} mg^{-1}. Abbreviations as in Table I.

	State 4	State 4 + C-atract.	State 3	State 3 + C-atract.
Oxidation rate in % of maximal state 3 oxidation	11.7	14.7	100	35.3
ADP consumption by oxidative phosphorylation (nmol/min × 2.3 mg)	0.0	0.0	552.0	147.2
M_p (%)	33.9	65.9	77.2	63.9
L (μm^{-1})	5.00 ± 2.01	6.26 ± 3.33 (n.s.)	5.76 ± 2.00 (n.s.) (n.s.)	6.94 ± 3.64 $P \leqslant 0.05$ (n.s.) (n.s.)
L_p (μm^{-1})	1.69 ± 0.68	4.12 ± 2.19 $P \leqslant 0.01$	4.44 ± 1.54 $P \leqslant 0.01$ (n.s.)	4.43 ± 2.32 $P \leqslant 0.01$ (n.s.) (n.s.)

TABLE VII

Effect of the membrane potential on contact formation

Mitochondria (18.9 mg/ml) were incubated in 113 mM sucrose, 10 mM K_2HPO_4 (pH 7.4), 5 mM $MgCl_2$, 12.5 mM succinate. Maximal oxidation rates were induced by addition of 5 μM valinomycin in the presence of 80 mM KCl. 10 mM ADP or ATP was added as indicated. The maximal state 3 activity of the mitochondria was 40 nmol O_2 min^{-1} mg^{-1}.

	Uncoupled oxidative phosphorylation		
	no addition	10 mM ADP	10 mM ATP
Oxidation rate in % of maximal state 3 oxidation	100	100	100
M_p (%)	29.2	58.8	38.7
L (μm^{-1})	5.65 ± 2.31	7.09 ± 2.69 (n.s.)	6.30 ± 3.30 (n.s.) (n.s.)
L_p (μm^{-1})	1.65 ± 0.68	4.17 ± 1.58 $P \leqslant 0.01$	2.44 ± 1.28 $P \leqslant 0.05$ $P \leqslant 0.01$

inhibition with carboxyatractyloside which also results in orientation of the translocator towards the outer surface of the inner membrane [18]. When we applied carboxyatractyloside in a concentration which almost completely inhibited the activity of the ATP/ADP exchange, the frequency of contacts remained high in the presence or absence of ADP (Table VI). Based on these data, it appeared that the binding of either ADP or carboxyatractyloside to the translocator induced contact formation.

Is ATP able to induce contact sites?

The data presented above suggested that binding of a ligand to the translocator may be responsible for contact formation. Therefore, we evaluated the possibility that ATP might have the same effect. In the presence of a membrane potential where ATP is a non-transportable ligand for the translocator, it was ineffective (Table III), as the contact formation was exclusively dependent on the turnover of ATP to ADP.

In view of these findings, we compared the function of ADP or ATP in contact formation in the absence of a membrane potential by abolishing it with K^+ and valinomycin. Under these conditions, ADP remained effective in inducing contacts, whereas ATP had a significantly lesser effect (Table VII).

Distribution of the adenylate translocator in subfractions of the mitochondrial membranes

Using electron microscopy and immuno-gold labelling we have observed that hexokinase in liver and brain mitochondria was bound to the surface preferentially where the two envelope membranes were attached [13, 19]. We have used these antibodies against hexokinase to precipitate the contact site fraction from disrupted mitochondrial membranes [14,19]. This contact fraction contained the inner envelope membrane and was characterized by high activity of hexokinase, nucleoside diphosphate kinase, and creatine kinase in brain mitochondria. The fraction could be separated from outer and inner membrane fragments also by density gradient centrifugation.

It is possible that the adenylate translocator might be involved in formation of contacts. To determine this we studied its distribution in the peripheral and crista forming part of the inner membrane by determining the binding of [^{3}H]atractyloside to fractions of inner and outer membranes and contact fraction obtained by density gradient centrifugation from osmotically disrupted kidney and liver mitochondria (Table VIII). Although we did not directly determine the effect of atractyloside in the fractions, we considered 11 nmol/mg as a saturating concentration. This was estimated from the observation in Table VI, where 0.8 nmol/mg carboxyatractyloside were sufficient to inhibit the oxidative phosphorylation to 75%. The data were normalized on both the protein content of the respective fractions and on cytochrome oxidase, which in liver mitochondria is found concentrated in the crista membranes [20].

The outer membrane fraction of liver and kidney mitochondria contained only small amounts of the adenylate translocator (not shown). In kidney mitochondria the translocator was not significantly enriched in the contact fraction. By contrast, in liver mitochondria the translocator appeared to be concentrated in the inner boundary membrane fraction relative to the distribution of the cytochrome oxidase. Our data indicated that the distribution of the translocator varies along the inner membrane of different mitochondria, but in general, it was present at the peripheral part of the inner membrane which performs the contact sites.

TABLE VIII

Distribution of the adenylate translocator in subfractions of mitochondrial membranes

Contact sites, and inner and outer membrane fractions were isolated as described in Ref. 14. The fractions were incubated in isolation medium in the presence of [^{3}H]atractyloside, centrifuged and analyzed for radioactivity.

	Inner membrane	Contact sites
Liver		
cpm/mg	14261	12616
cpm/nmol cyt ox	33114	90114
Kidney		
cpm/mg	3392	2664
cpm/nmol cyt ox	4978	3232

Summary

Table IX is provided to summarize the large amount of data that have lead us to conclude that only ADP and carboxyatractyloside, and not ATP are able of inducing contacts. The results given here also show that the activities of electron transport, ATP synthesis and membrane potential have no regulatory function in contact formation.

Discussion

Effect of uncouplers on contact formation

The effect of uncouplers on the formation of contact sites is diverse. We previously observed a reduced frequency of contacts in the presence of DNP [7] and free fatty acids [11]. In both cases we were unable to induce contacts by addition of ADP. Conversely, as documented in Table VII, contacts could be induced by ADP although the mitochondria were uncoupled by K + and valinomycin. This suggests that the increase in negative surface charge caused by 50 μM DNP and as shown recently by 30 μM fatty acids [21] might interfere with the ADP induced contact formation.

Mitochondrial volume changes and contact formation

Small amplitude mitochondrial volume changes have been previously described in conjunction with the transition upon addition of ADP from the controlled to the active respiratory state [22,23]. These structural changes were not related to the orthodox/condensed transformation defined by Hackenbrock [8,9] because they were not linked to active electron transport. Stoner and Sirac [24,25] observed that ADP- and ATP-induced shrinkage of the mitochondria required very low concentrations of these metabolites and was independent of the energy transfer to the nucleotides. Moreover, Scherer and Klingenberg [18] employing specific inhibitors, reported that the expanded or contracted states were associated with the orientation of the adenine nucleotide translocator at the outer or inner surface of the inner membrane.

TABLE IX

Factors regulating contact site formation

	Effective	Ineffective	Refers to Table
ADP	+	–	II, IV, VII
Carboxy-atractyloside	+	–	VI
ATP	–	+	III, VII
Membrane potential	–	+	VII
Electron transport	–	+	II
Activity of ATPase	–	+	V

Collectively these results suggested that the binding of adenine nucleotides to the mitochondrial surface caused structural changes in the mitochondrial membranes which were independent of the electron transport and activity of mitochondrial ATPase.

The structural changes, which were visualized by conventional electron microscopy [24] or turbidity measurements [25,18], are in good agreement with our results obtained by freeze fracture. According to earlier investigations the fracture plane deflections represent contact sites between the two envelope membranes [2,7,26]. In agreement with Stoner and Sirac [24] our quantification of the fracture plane "jumps" or contacts demonstrated that their formation was not related to electron transport (Table II) or ATPase activity (Table V). Rather, the frequency of contacts correlated very well with the concentration of free ADP (Tables III and IV). Conversely, in *energized* mitochondria the addition of 5 mM ATP (Table III) did not induce contact frequency .

In order to gain insight into the mechanism of the ADP effect we applied atractyloside to the mitochondria a compound which inhibits ADP-induced contraction [18,25]. In contrast to previous investigations of Stoner and Sirac [25] and Scherer and Klingenberg [18] carboxyatractyloside induced contact formation Table VI.

Structural changes of the adenylate translocator and contact formation

If conformational changes of the adenylate translocator are involved in the contact formation, the model concerning the conformational states of the ATP/ADP carrier presented by Vignais et al. [27] would provide the best interpretation of our results. These authors postulate two binding sites for ADP one of which overlaps with the carboxyatractyloside binding site.

This model could explain our results showing that ADP and carboxyatractyloside had the same effect. According to these authors, binding of transportable nucleotides to high affinity binding sites induces low affinity transport sites, whereas non-transportable nucleotides are not effective. Since ATP, in the presence of a membrane potential, would be a non-transportable ligand it should not induce conformational changes of the ATP/ADP carrier. On the contrary one might expect an ATP effect in the absence of a membrane potential. This did not occur as, after depletion of the membrane potential, ATP still remained ineffective to induce contacts (Table VII).

Regulatory aspects of the carrier induced contact formation

The adenylate translocator was found to be located in the peripheral part of the inner membrane (Table VIII). Thus, interaction with the outer membrane is possible most likely with the mitochondrial porin. This suggestion is supported by experiments showing that addition of porin to mitoplasts enhanced the carboxyatractyloside sensitivity of ADP-stimulated respiration [28]. The controversial discussion of this observation [29,30] leads to the additional information that the effect of porin was higher in mitoplasts derived from mitochondria with low respiratory control. This aspect would agree with our ideas, as such mitochondria exhibit a reduced number of contacts and less contamination by outer membrane. It has also been observed that peripheral kinases which bind specifically to mitochondrial porin such as hexokinase and glycerolkinase [31,32,33] have preferential access to intramitochondrial ATP [34,33].

Such compartmentation of ATP and ADP between the translocator and these kinases would improve the exchange of mitochondrial energy [6]. The cytosolic steady state concentration of ADP in intact liver cells [35] is approx. 500 μM and 20-times lower in muscle [36]. In order to sustain a high phosphorylation potential in the cytosol the ATP/ADP quotient of 15 in liver and 200 in muscle does not vary much during activity [37]. Under these conditions the question arises as to how mitochondrial respiratory activity is regulated without significant fluctuations of the free cytosolic ADP. With the proposed interaction between the two membranes, the activity of ATP consuming kinases at the surface of the inner membrane could provide a promising mechanism to increase the free ADP concentration in the outer mitochondrial compartment. This functional coupling with kinases such as hexokinase, creatine kinase, and nucleoside diphosphate kinase of the ATP/ADP translocator would displace the transport system from equilibration. The latter aspect is important as, according to in vitro measurements [38], the electrogenic ATP export generates an ATP/ADP ratio of 15–20, which is equivalent to or lower than that in the cytosol.

These considerations led us to conclude that the contact formation under physiological conditions is controlled by the activity of peripheral kinases via a local elevation in the ADP concentration in the outer mitochondrial compartment. Thus, the activity of oxidative phosphorylation would be controlled by the level of the kinase substrates (such as glucose in liver and/or creatine in muscle and brain tissue) rather than by free ADP.

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References

1 Hackenbrock, C.R. (1968) Proc. Natl. Acad. Sci. USA 61, 598–605.

2 Van Venetie, R. and Verkleij, A.J. (1982) Biochim. Biophys. Acta 692, 397–405.

3 Cremers, F.F.M., Voorhout, W.F., Van der Krift, T.P., Leunissen-Bijvelt, J.J.M. and Verkleij, A. (1988) Biochim. Biophys. Acta 933, 334–340

4 Schleyer, M. and Neupert, W. (1985) Cell 43, 339–350.

5 Pon, L., Moll, T., Vestweber, D., Marshallsay, B. and Schatz, G. (1989) J. Cell Biol. 109, 2603–2616

6 Brdiczka, D. Adams, V., Kottke, M. and Benz, R. (1989) in Anion carriers of mitochondrial membranes (Azzi A., Nałecz, K.A., Nałecz, M.J. and Wojtczak, L., eds.), pp.361–372, Springer, Heidelberg.

7 Knoll, G. and Brdiczka, D. (1983) Biochim. Biophys. Acta 733, 102–110.

8 Hackenbrock, Ch.R. (1966) J. Cell Biol. 30, 269–297

9 Hackenbrock, Ch.R. (1968) J. Cell Biol. 37, 345–369.

10 Riesinger, I., Knoll, G., Brdiczka, D., Denis-Pouxviel, C., Murat, J.C. and Probst. I. (1985) Eur. J. Cell Biol. 36, 53.

11 Klug, G., Krause, J. Östlund, A.K., Knoll, G. and Brdiczka, D. (1984) Biochim. Biophys. Acta 764, 272–282.

12 Denis-Pouxviel, C., Riesinger, I., Bühler, C.,Brdiczka, D. and Murat,J-C. (1987) Biochim. Biophys. Acta 902, 335–348.

13 Weiler, U., Riesinger, I., Knoll, G. and Brdiczka, D. (1985) Biochem. Medicine 33, 223–235.

14 Adams , V., Bosch, W.,Schlegel, J., Wallimann, T. and Brdiczka, D. (1989) Biochim. Biophys. Acta 981, 213–225.

15 Estabroock, R.W. (1967) Methods Enzymol. 10, 41–47.

16 Knoll, G., Oebel, G. and Plattner, H. (1982) Protoplasma 111, 161–176

17 Branton, D.S., Bullivant, S., Gilula, N.B., Karnovsky, M.J., Moor, H., Muehlethaler, K., Northcote, D.H., Packer, L., Satir, B., Speth, V., Staehelin, L.A., Steere, R.L. and Weinstein, R.S. (1975) Science 190, 54–56.

18 Scherer, B. and Klingenberg, M. (1974) Biochemistry 13, 161–170

19 Kottke, M., Adams, V., Riesinger, I., Bremm. G., Bosch, W., Brdiczka, D., Sandri, G. and Panfili, E. (1988) Biochim. Biophys. Acta 395, 807–832.

20 Brdiczka, D., Reith, A. (1987) in The organization of cell metabolism (Welch, G.R. and Clegg, J.S., eds.), pp. 277–287, Plenum, New York.

21 Wojtczak, L., Adams, V. and Brdiczka, D. (1988) Mol. Cell. Biochem. 79, 25–30.

22 Bücher, Th. and Klingenberg, M. (1958) Angew. Chem. 70, 552–570

23 Packer, L. (1960) J. Biol. Chem. 235, 242–249

24 Stoner, C.D. and Sirak, H.D. (1973) J. Cell Biol. 56, 51–64

25 Stoner, C.D. and Sirak, H.D. (1973) J. Cell Biol. 56, 65–73

26 Ohlendieck, K., Riesinger, I., Adams, V., Krause, J. and Brdiczka, D. (1986) Biochim. Biophys. Acta 860, 672–689.

27 Vignais, P.V., Brandolin, G., Boulay, F., Dalbon, P., Block, M.R. and Gauche, L. (1989) in Anion carriers of mitochondrial membranes (Azzi A., Nałecz, K.A., Nałecz, M.J. and Wojtczak, L., eds.), pp.133–146, Springer, Heidelberg.

28 Pronevich, L.A., Mirzabekov, T.A. and Rozhdestvenskaya, Z.E. (1989) FEBS Lett. 247, 330–332.

29 Wojtczak, L. and Nałecz, M. (1990) FEBS Lett. 269, 149.

30 Pronevich, L.A., Mirzabekov, T.A. (1990) FEBS Lett. 269, 150–151.

31 Fiek, Ch., Benz, R., Roos, N. and Brdiczka, D. (1982) Biochim. Biophys. Acta 688, 429–440.

32 Östlund, A.K., Göhring, U., Krause, J. and Brdiczka, D (1983) Biochem. Med. 30, 231–245.

33 Brdiczka, D., Knoll, G., Riesinger, I., Weiler, U., Klug, G., Benz,

R. and Krause, J. (1986) Myocardial and skeletal muscle bioenergetics (Brautbar, N., ed.), pp.55–69, Plenum, New York.

34 Gots, R.E. and Bessman, S.P. (1974) Arch. Biochem. Biophys. 163, 7–14.

35 Soboll, S., Scholz, R. and Heldt, H.W. (1978) Eur. J. Biochem. 87, 377–390.

36 Hebisch, S., Sies, H. and Soboll, S. (1986) Pflügers Arch. 406, 20–24.

37 Ackerman, J.J.H., Grove, T.H., Wong, G.G., Gadian, D.G. and Radda, G.K. (1980) Nature 283, 167–170.

38 Klingenberg, M. and Heldt, H.W. (1982) Metabolic Compartmentation (Sies, H., ed.), pp.101–122, Academic Press, New York.