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EXCHANGE OF ADENINE NUCLEOTIDES IN DIGITONIN-TREATED MITOCHONDRIA

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SUMMARY

1. The exchange of adenine nucleotides has been studied in phosphorylating inner membrane preparations of rat-liver mitochondria prepared by treatment with digitonin.

2. The digitonin treatment removes nearly 75 % of the outer membrane, as seen by the loss of rotenone-insensitive NADH cytochrome *c* reductase, while the activity of malate dehydrogenase and the concentration of adenine nucleotides are slightly increased.

3. The exchange of adenine nucleotides is selective towards ADP and ATP, and is completely sensitive to atractyloside. *m*-Chlorocarbonylcyanide phenylhydrazone stimulates the ATP exchange, and concomitantly inhibits the P_i–ATP exchange.

4. The digitonin particles are considerably more susceptible to changes in osmolarity than intact mitochondria, and exhibit no sucrose-impermeable space at 0.5 M sucrose. Furthermore, the exchange of ADP is completely inhibited.

5. This study provides direct evidence that the inner membrane is the site of adenine nucleotide binding, and that the properties of these sites are unaffected by the digitonin treatment.

INTRODUCTION

Isolated mitochondria are known to exchange endogenous adenine nucleotides readily with external ADP and ATP, but not with other purine or pyrimidine nucleotides. Although most evidence suggests the existence of a translocating enzyme that catalyzes an obligatory exchange of intra- and extra-mitochondrial ADP and ATP[1–4], the data has also been interpreted as a simple binding of nucleotides to certain sites on the membrane that are directly responsible for ADP phosphorylation[5,6]. Atractyloside has been found to inhibit the exchange[7,8] presumably by preventing the binding of exogenous adenine nucleotides to sites on the mitochondrial membrane[4,9]. From studies with ^{14}C-labeled sucrose and dextran, it has been postulated that the outer membrane of the mitochondrion is freely permeable to nucleotides, and that the inner membrane is the site of the adenine nucleotide exchange[8].

This study directly tests this theory by examining the adenine nucleotide ex-

Abbreviation: CCCP, *m*-chlorocarbonylcyanide phenylhydrazone.

change system in inner membrane preparations obtained by treatment of rat-liver mitochondria with digitonin, which results in submitochondrial particles lacking outer membrane, but possessing normal oxidative phosphorylation and P_i–ATP exchange[10]. Results of this study provide direct evidence that the inner membrane is the site of adenine nucleotide binding and that the properties of these sites are unaffected by the digitonin treatment.

METHODS

Rat-liver mitochondria were isolated in a medium containing 0.25 M sucrose–1 mM EDTA–20 mM triethanolamine buffer (pH 7.2), and washed twice in 0.3 M sucrose, care being taken to remove all the top fluffy layer.

The outer membrane was removed according to HOPPEL AND COOPER[10] by treatment for 5 min at 0° with 1.1 mg digitonin per 10 mg mitochondrial protein, the final concentration of digitonin being 1 %. The preparations were either used fresh or after freezing at −80° in 15 % anhydrous dimethylsulfoxide.

To measure the adenine nucleotide exchange, the mitochondrial adenine nucleotide pool was first labeled with 0.5 μC [^{14}C]ATP (ADP) and 0.2 mM ATP (ADP) for 20 min, 0°, followed by two washings in 0.3 M sucrose. The rate of adenine nucleotide exchange was determined by two methods, both involving incubation of prelabeled mitochondria with exogenous unlabeled nucleotide and counting the appearance of radioactivity in the supernatant[11]. In the first method, the exchange of adenine nucleotides was measured by stopping the reaction with 50 μM atractyloside and the particles immediately separated from the medium in an Eppendorf microcentrifuge at 13500 rev./min for 45 sec. The second method involved a filtration centrifugation at 25000 rev./min in a SW-50L rotor through silicone oil (Dow 550, specific gravity 1.063) and into $HClO_4$ (refs. 12 and 13).

To measure the ^{14}C-labeled adenine nucleotides released from the mitochondria, an aliquot of a neutralized extract of the supernatant or sediment was pipetted onto glass fiber discs, dried, and counted by liquid scintillation in an 0.4 % 2,5-diphenyloxazole–toluene mixture. Addition of various amounts of the secondary fluor, 1,4-bis-(2-(4-methyl-5-phenyloxazolyl)), slightly lowered the counting efficiency and was therefore not used in this system.

Compartmentation of whole mitochondria and inner membrane–matrix preparation was measured by the isotopic dilution method[14] employing freshly prepared samples containing 20–25 mg protein in 2.5 mM triethanolamine (pH 7.0) *plus* sucrose to the required molarity. Centrifugation was performed in 0.8-cm glass tubes for 5 min at 20000 rev./min, 0°. Aliquots of [^{14}C]dextran (mol. wt. 60000–90000) or [^{14}C]-sucrose-labeled supernatant and original solution were plated onto glass fiber discs and counted as described for ^{14}C-labeled adenine nucleotides.

Protein was determined by a biuret method, using KCN to eliminate interference by phospholipids[15].

The content of mitochondrial adenine nucleotides was determined enzymatically[16]. Measurement of NADH–cytochrome *c* reductase (rotenone insensitive), adenylate kinase, and malate dehydrogenase activity were performed exactly as described by HOPPEL AND COOPER[10]. The P_i–ATP exchange was measured in 0.5 ml containing 10 mM ATP, 0.1 % bovine serum albumin, 20 mM triethanolamine (pH 7.2),

10 mM P_i containing 10^6 counts/min ^{32}P, and 3.0 mg protein. The P_i–ATP exchange was corrected for the oligomycin-insensitive blank.

RESULTS

Enzyme and nucleotide content

Recent studies have indicated that the characteristic enzymes of the outer membrane of mitochondria are monoamine oxidase[17] and NADH–cytochrome *c* reductase (rotenone-insensitive)[10,18] while the inner membrane–matrix contains Krebs-cycle enzymes[17,18], enzymes of fatty acid oxidation[19] and various nucleotides[20]. With these as markers, Table I shows that treatment with digitonin has removed 72 % of the outer membrane, as seen by the loss of the rotenone-insensitive NADH–cytochrome *c* reductase. The content of malate dehydrogenase and total adenine nucleotides is slightly higher, an enrichment caused by the removal of the outer membrane which presumably is devoid of both malate dehydrogenase and adenine nucleotides. Table I also shows that adenylate kinase, thought to be localized between the inner and outer membrane[8] is 96 % removed in these digitonin preparations. The removal of this enzyme by digitonin is particularly important in studying the exchange of exogenous ADP, as transphosphorylation of ADP to AMP *plus* ATP is kept to a minimum.

TABLE I

EFFECT OF DIGITONIN ON MITOCHONDRIAL ADENINE NUCLEOTIDE CONTENT AND ENZYME ACTIVITY

	Whole mitochondria	*Inner membrane–matrix*	% *Loss (gain)*
	nmoles/mg protein		
Σ ATP, ADP, AMP	14.82	15.64	(5.5)
	μmoles/mg protein per min		
Malate dehydrogenase	4.70	4.75	(1.0)
NADH–cytochrome *c* reductase (rotenone-insensitive)	0.291	0.080	72.5
Adenylate kinase	0.896	0.036	96.0

Characteristics of the nucleotide exchange

Table II shows that the ^{14}C-labeled adenine nucleotide pool in the inner membrane preparation exhibits a high specificity towards exchange by external ADP and ATP and is insensitive to AMP and GDP. The percent exchange of ADP at 30 sec is 49 %, that of ATP 15 %, while AMP and GDP have negligible exchanges. Inclusion of 50 μM atractyloside with the unlabeled nucleotides completely suppresses the exchange in all cases.

Intact rat-liver mitochondria show a K_m for the exchange of ADP and ATP of 5–10 μM (ref. 11). This study has measured the exchange in digitonin-treated inner membrane preparations as a function of nucleotide concentration, and Lineweaver–Burk plots reveal a K_m of 9.0 and 8.5 μM for ADP and ATP, respectively, and a v_{max} of 6.2 and 2.6 nmoles per min per mg protein.

The exchange of ATP in whole mitochondria may be increased by the addition of an uncoupler of oxidative phosphorylation, such as *m*-chlorocarbonylcyanide phenylhydrazone (CCCP), while the exchange of ADP is not affected[11]. The relation between the degree of coupling of oxidation to phosphorylation, as measured by the P_i–ATP exchange, and the exchange of ATP as a function of the concentration of CCCP, is demonstrated for the inner membrane preparation in Fig. 1. The rate of the P_i–ATP exchange at 10° in the absence of CCCP is 12.7 nmoles ATP per min per mg, which compares with 21.7 nmoles ATP per min per mg found in whole mitochondria at 10°. The greater activity of the P_i–ATP exchange than the ATP exchange is probably due to the hypotonic conditions employed in the P_i–ATP exchange assay. It is known that the phosphorylation reactions are markedly stimulated by decreasing the sucrose concentration[21]. Addition of CCCP decreases the P_i–ATP exchange and simultaneously causes an increase in the ATP translocation, with a half-maximal effect on both exchanges occurring at 0.06–0.08 μM CCCP. Although not shown in

TABLE II

SPECIFICITY OF THE ADENINE NUCLEOTIDE EXCHANGE IN DIGITONIN PARTICLES

The adenine nucleotide pool in digitonin particles was prelabeled with [^{14}C]ADP. The back exchange was measured by filtration layer centrifugation at 0° with 0.2 mM nucleotide, and, where indicated, 50 μM atractyloside. Protein, 4.2 mg in 0.5 ml; time of exchange, 30 sec; extra-mitochondrial ^{14}C-labeled adenine nucleotide was corrected for by [^{14}C]dextran.

Additions	*Atractyloside*	*Exchange* (%)
ADP	—	49.2
ADP	+	0
ATP	—	14.9
ATP	+	0
AMP	—	2.0
AMP	+	0
GDP	—	0.2
GDP	+	0

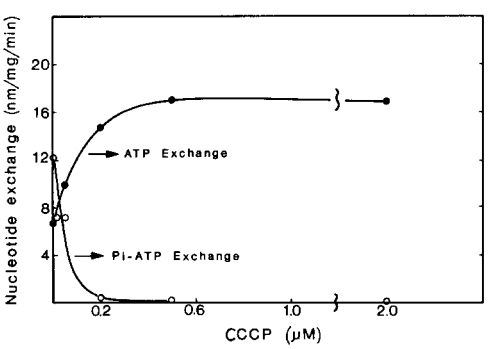

Fig 1. Effect of CCCP on ATP and P_i–ATP exchange in digitonin particles The exchange of 0.2 mM ATP was measured for 15 sec at 10° in [^{14}C]ATP-loaded digitonin particles, the reaction stopped by addition of 50 μM atractyloside, and the particles immediately separated in a microcentrifuge. The total ATP exchange was subtracted from the exchange in controls incubated with 50 μM atractyloside and ATP. P_i–ATP exchange was determined as in METHODS.

Fig. 1, the P_i–ATP exchange and ATP exchange were completely blocked by 50 μM atractyloside.

Water content of digitonin particles

As is well known, isolated mitochondria behave as rather imperfect osmometers, swelling and shrinking as the osmolarity is changed[14,22]. By employing ^{14}C-labeled sucrose and dextran, it is possible to determine the existence of three compartments in intact mitochondria; a sucrose-impermeable inner membrane–matrix space, a sucrose-permeable space between the inner and outer membrane, and a dextran-permeable extramitochondrial space[8,12].

In Figs. 2A and 2B, an isotopic dilution method[14] using [^{14}C]sucrose and [^{14}C]-dextran has been employed to examine the compartmentation in digitonin-treated inner membrane preparations compared to whole mitochondria. It should be noted first that the extramitochondrial water content (gravimetrically-determined total water–sucrose permeable and impermeable space) exhibits a minimum at 0.2–0.3 M sucrose for whole mitochondria and digitonin particles and becomes greater as the osmolarity is raised or lowered. A trivial, but likely explanation for the greater extramitochondrial space under hypertonic conditions is that the time and speed at which centrifugation occurred was insufficient to allow maximum packing of the "cretinated" particles. This increase in total water (or extramitochondrial space) at high osmolarities was not expected, and might explain the apparent discrepancy found by HARRIS AND VAN DAM[23] between mitochondrial volume changes measured by light scattering or sucrose permeability.

The behavior or the "intra"-mitochondrial water (sucrose permeable and impermeable spaces) towards changes in osmolarity is quite different between the two preparations.

A doubling of the osmolarity from 0.1 to 0.2 M decreases the sucrose-imper-

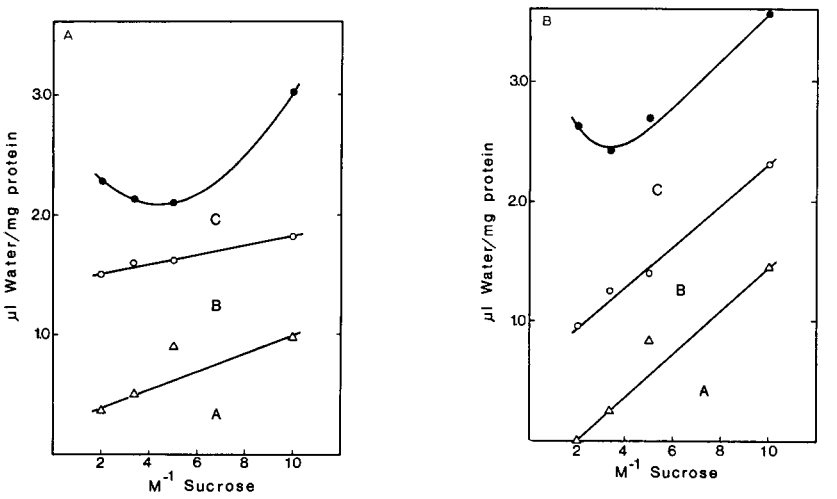

Fig. 2. Permeability of whole rat-liver mitochondria (A) and digitonin-treated inner membrane preparation (B). "A" refers to the sucrose-impermeable space; "B" is the sucrose-permeable space, and "C" is the dextran-permeable space. Each point represents a mean of either 4 (A) or 6 (B) samples.

meable space (A) in intact mitochondria (Fig. 2A) from 1.00 to 0.61 μl per mg protein, while the sucrose-permeable space (B) increases from 0.81 to 1.01 μl per mg protein. These results are in line with the known behavior of the cristae membrane as an osmometer, contracting and unfolding with changes in osmotic pressure[14], and of the outer membrane as a freely permeable structure being little affected by osmotic pressure[24]. The impermeable space in the inner membrane preparations is considerably more subject to osmolarity, decreasing from 1.42 to 0.54 μl/mg as the sucrose concentration is increased from 0.1 to 0.2 M. At the most hypertonic sucrose concentration, 0.5 M, Fig. 2B shows that there is no intramitochondrial space, while the sucrose-permeable space (B) exhibits no change over the entire range of osmolarity. Inasmuch as the inner membrane preparation should have no sucrose-permeable space, the constancy of the space over a wide range of osmolarity suggests that a certain percentage of the particles may be disrupted, and thus completely permeable to sucrose (*cf.* ref. 25).

Effect of osmolarity on nucleotide exchange

Table III reveals that incubation of freshly prepared particles in 0.5 M sucrose caused a 20% loss of adenine nucleotides, from 9.4 to 7.5 nmoles per mg protein, but a nearly complete suppression of the exchange of unlabeled external ADP with endogenous ^{14}C-labeled nucleotides.

TABLE III

EFFECT OF OSMOLARITY ON ADP EXCHANGE IN DIGITONIN PARTICLES

Freshly prepared particles were incubated in 0.25 mM [^{14}C]ADP for 15 min, 0°, and washed twice at 11000 rev./min. The back exchange in 0.5 mM ADP was measured at 0° for 15 sec by the atractyloside-stop method. Controls were incubated in the presence of 50 μM atractyloside *plus* ADP and this atractyloside-insensitive exchange subtracted from the total exchange. Adenine nucleotide content was measured enzymatically and corrected for leakage of ^{14}C label.

Osmolarity (M)	*Adenine nucleotide content (nmoles/mg)*	*ADP exchange (nmoles ADP per min per mg)*
0.1	9.4	6.7
0.5	7.5	0.4

DISCUSSION

These results support the contention that the inner membrane of rat-liver mitochondria is the site of the exchange of adenine nucleotides, as proposed by KLINGENBERG AND PFAFF[8] on the basis of permeability studies with [^{14}C]dextran and sucrose, and confirmed for ADP by WINKLER *et al.*[4] in digitonin-treated mitochondria. Recent work has demonstrated that the inner mitochondrial membrane is also the site of a partially atractyloside-sensitive ADP–ATP exchange[26]. In the present study, although nearly 75% of the outer membrane was removed in the digitonin preparations, the rate of the adenine nucleotide exchange per mg protein is equal to that found in intact mitochondria, suggesting that the outer membrane contains no binding sites capable of participating in the exchange of ADP and ATP. The data also indicate

that the properties commonly associated with the adenine nucleotide exchange in intact mitochondria, namely the selectivity of endogenous adenine nucleotides towards exchange with external ADP and to a lesser extent ATP, and the atractyloside sensitivity of the exchange, have been retained in the digitonin-prepared inner membrane fraction. In a more disorganized system such as sonicated mitochondria[3,6,27], the sensitivity towards atractyloside is lost, and the oxidative phosphorylation system exhibits a non-specificity toward phosphate acceptor.

It must be cautioned that the exchange may not be totally selective for the adenine nucleotides even under optimal conditions. In all the reported experiments with intact mitochondria[3,4,11], the back exchange of ^{14}C-labeled adenine nucleotides from mitochondria in the presence of unlabeled nucleotides has been measured, and negative values therefore only indicate that external nucleotide is not exchanged for internal ^{14}C-labeled adenine nucleotides. A small binding or exchange of [^{14}C]GDP in rat-liver mitochondria has recently been reported[28] and under certain conditions it is even possible to cause an influx of 20–50 nmoles [^{14}C]GDP per mg protein (unpublished result). Indeed, the presence of DNA within the matrix space makes the transport of non-adenine nucleotides likely, although the rate of binding may be considerably slower, and not influenced by atractyloside.

The low exchange of ATP compared to ADP is thought to be a characteristic of a coupled oxidative phosphorylation system[11]. The enhancement of the ATP exchange and simultaneous decrease of the P_i–ATP exchange by addition of CCCP to the preparation suggests that not only are the digitonin particles coupled, but also that there is a common point of action of the uncoupler. This has been postulated to be the collapse of the membrane potential by the uncoupler[11], leading to a higher rate of exchange diffusion of the ATP^{4-} molecule against a less negative internal electrochemical gradient.

The greater responsiveness of the digitonin preparation to changes in osmotic pressure emphasizes the protective role of the outer membrane in preventing large amplitude volume changes from occurring *in situ*. It also points out that other swelling phenomena such as energy-linked ion transport or antibiotic-mediated ion diffusion may also be of greater magnitude in the digitonin particles. In favor of this is the recent finding that ATP or succinate-induced changes in light scattering mediated by gramicidin in the presence of Na^+ or K^+ are greater in digitonin particles than in intact mitochondria (C. E. WENNER AND H. M. MEISNER, unpublished).

It is significant that, at 0.5 M sucrose, there is no measurable sucrose-impermeable space (*i.e.*, inner membrane space) in digitonin-treated mitochondria. It follows that all organic and inorganic ions in these particles are bound and not osmotically active. The adenine nucleotide content at 0.5 M sucrose is nearly normal, yet these endogenous nucleotides are not capable of being exchanged with external nucleotides. It has been known for some time that sucrose and other polyols severely inhibit the rate of oxidation[29], and the data reported here lends evidence to the suggestion by LEHNINGER[30] that sucrose also prevents the binding of nucleotides to exchange sites on the membrane.

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