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THE ELECTROGENIC NATURE OF ADP/ATP TRANSPORT IN INSIDE-OUT SUBMITOCHONDRIAL PARTICLES

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Summary

1. Beef heart submitochondrial particles prepared by sonication loaded with ADP and $MgCl_2$ are competent in ADP/ATP transport. The $\Delta\psi$ and ΔpH developed by these particles have been determined, and the electrogenic nature of the ADP/ATP transport that they catalyze has been assessed.

2. $MgCl_2$ particles, prepared by sonication, oxidizing succinate in the presence of oligomycin develop a $\Delta\psi$ of 95 mV (positive inside) and a ΔpH of 1.5 pH units (acidic inside), as measured by the distribution of $S^{14}CN^-$ ($\Delta\psi$) and [^{14}C]methylamine (ΔpH) using the flow dialysis technique. The $\Delta\psi$ values determined with the thiocyanate electrode are somewhat higher (110—130 mV). $\Delta\psi$ and ΔpH are partly interchangeable.

3. The fluorescent dye, dipropylthiodicarbocyanine, and the absorbing dye, neutral red, are suitable probes for continuous, qualitative monitoring of $\Delta\psi$ and ΔpH respectively. The $\Delta\psi$ and ΔpH responses to respiratory activity in particles prepared by sonication are opposite to those found with intact mitochondria, in agreement with the reverse polarity of the membrane in particles prepared by sonication.

4. ATP added to oligomycin-pretreated particles prepared by sonication is taken up in exchange for internal ADP. Under these conditions, the fluorescence intensity of dipropylthiodicarbocyanine is transitorily quenched, which corresponds to the net influx of an excess of negative charges inside the particles. A subsequent addition of ADP results in a transient rise in fluorescence.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Mops, 3-(*N*-morpholino)-propanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

The ATP-induced fluorescence quenching of dipropylthiocarbocyanine is abolished by addition of SCN^-, a strong permeant anion which collapses the $\Delta\psi$. It is inhibited by ADP in a competitive manner. This fluorescence quenching is specific for ATP among a number of nucleotides tested, is inhibited by bongkrekic acid and is highly dependent on temperature. All these features are typical properties of the ADP/ATP carrier.

5. Assays with neutral red as a pH indicator show that the internal phase of particles prepared by sonication treated by oligomycin becomes more acidic upon addition of ATP. However the pH changes are markedly diminished at high concentration of K^+ and totally abolished by K^+ plus valinomycin and, on the contrary, enhanced by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. This suggests that the ATP-dependent changes of pH are not characteristic of the mechanism of the ADP/ATP carrier, but correspond to a passive transport of H^+.

6. In respiring particles prepared by sonication, addition of SCN^- at a concentration of 5 mM, sufficient to collapse the $\Delta\psi$, strongly modifies the kinetics of $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange, but not that of $ADP^{3-}_{ex}/ADP^{3-}_{in}$. It increases the K_m value for ATP_{ex} and decreases the maximal velocity of transport. Nigericin has no effect.

7. In non-respiring particles prepared by sonication, addition of a sufficiently high concentration of SCN^- (30 mM) induces a transient diffusion potential which results in the inhibition of $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange. When the excess of negative charges carried inside the particles by SCN^- are neutralized by the simultaneous entry of K^+, the $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange remains unaltered.

8. The above data indicate that ADP/ATP transport across the mitochondrial membrane is primarily electrogenic.

Introduction

Inside-out submitochondrial particles obtained by sonication of beef heart mitochondria in the presence of ADP (or ATP) and $MgCl_2$ are competent in specific transport of ADP and ATP by exchange-diffusion. Their kinetic properties and the asymmetric binding of two specific inhibitors, atractyloside (or carboxyatractyloside) and bongkrekic acid, have been described [1—4]. As the polarity of their membrane is opposite to that of mitochondria [5], they offer the possibility to distinguish between the alternatives which were suggested for ADP/ATP transport in intact mitochondria. One of them was that the transport depends more on membrane potential ($\Delta\psi$) than on pH gradient (ΔpH) [6—11]. The assessment of the electrogenic nature of the mitochondrial ADP/ATP transport, using ADP-loaded sonic particles as biological material, required preliminary assays to check whether these particles are able to develop a significant $\Delta\psi$ and (or) ΔpH. Values of $\Delta\psi$ and ΔpH reported so far for particles prepared by sonication are in fact scattered and sometimes controversial [12—18]. The present study uncludes two series of experiments. In the first one, the values of ΔpH and $\Delta\psi$ in ADP-loaded particles prepared by sonication were critically assessed by different techniques, including the use of fluorescent and radioactive probes. In the second series of experiments, we

made use of the above assays to determine whether the ATP_{ex}/ADP_{in} exchange in particles prepared by sonication is electrogenic or is charge-compensated by H^+ movement. The evidence is in favour of the electrogenic nature of the ADP/ATP transport. Some of the data presented here have appeared in a preliminary form [19].

Methods

Submitochondrial particles, competent for ADP/ATP transport were prepared by sonication of beef heart mitochondria after dilution to a concentration of 10 to 15 mg protein/ml in 0.25 M sucrose, 3 mM Tris buffer, 15 mM $MgCl_2$, 10 mM ADP and 5 μg oligomycin/mg protein, final pH 7.4 [2]. The particles contained 3 to 5 nmol of ADP plus ATP per mg protein which corresponded to a concentration of ADP plus ATP of about 5 mM based on an internal space of 0.8 μl per mg protein. The internal space of particles was calculated from the difference between the volumes occupied by ^3H_2O (total space) and by [^{14}C]dextran (external space) in a pellet of particles. Adenine nucleotide transport was assayed by the back exchange technique with particles preloaded with [^{14}C]ADP (cf. ref. 2).

$\Delta\psi$ (positive inside) and ΔpH (acidic inside) in sonic particles were developed upon oxidation of succinate in the presence of oligomycin or by hydrolysis of ATP. ΔpH changes were monitored with 9-amino acridine [20] or neutral red [21,22] and $\Delta\psi$ changes by the fluorescent dye 3,3'-dipropylthiodicarbocyanine [23,24]. While fluorescent and colored probes may provide useful information by qualitative monitoring of $\Delta\psi$ and ΔpH, they are not generally accepted to be quantitative. Ion distribution techniques, based on the use of radioactively labeled ions, are considered to be more reliable [25]. In the present study on sonic particles, the strong permeant anion $S^{14}CN^-$ and the weak-base [^{14}C]methylamine were used for determination of $\Delta\psi$ and ΔpH respectively, following the same principle as that described by Schuldiner et al. [26] for bacterial chromatophores. $\Delta\psi$ was calculated from the distribution of $S^{14}CN^-$ inside and outside the particles by ther Nernst equation:

$$\Delta\psi = \frac{RT}{ZF} \ln \frac{[S^{14}CN^-_{in}]}{[S^{14}CN^-_{out}]} .$$

ΔpH was calculated from the distribution of [^{14}C]methylamine by the relation:

$$\Delta pH = \log \frac{[[^{14}C]\text{methylamine}_{in}]}{[[^{14}C]\text{methylamine}_{out}]} .$$

The radioactivity uptake by the particles was assayed by the flow dialysis technique first described by Colowick and Womack [27], and improved for higher sensitivity on the basis of theoretical considerations by Remy and Buc [28] and by Tenu et al. [29,30]. The dialysis cell had a diffusion area of 0.5 cm^2, a lower chamber of 0.2 ml and an upper chamber of 2 ml. A piece of dialysis tubing (Union Carbide) was inserted between the two chambers. The contents of both chambers were mixed by means of small magnetic stirring bars. Sonic particles were placed in the upper chamber and the medium was pumped through the

lower chamber at a rate of 4 ml/min. Energization of particles was induced by addition of succinate, as oxidizable substrate. To avoid anaerobiosis due to the high respiratory activity of particles prepared by sonication, the medium which was pumped through the lower chamber was supplemented with 0.2 M H_2O_2, and an amount of catalase able to convert 40 μmol of H_2O_2 to O_2 per min was added to the particle suspension in the upper chamber. The small percentage of H_2O_2 which diffused into the upper chamber was sufficient to generate a continuous flow of O_2 in the particle suspension. 1 ml fractions were collected in vials for scintillation counting.

We also measured the $\Delta\psi$ in sonic particles by monitoring the SCN^- concentration in the incubation medium by an Orion SCN^- electrode using an Orion ionalyzer model 901 connected to a Sefram Servotrace recorder.

$KS^{14}CN^-$ was obtained from Amersham and [^{14}C]methylamine from C.E.A. Saclay. Solutions of high specific radioactivities were used for flow dialysis assays. The specific radioactivity of the stock solution of $KS^{14}CN^-$ was 59 Ci/mol; that of the stock solution of [^{14}C]methylamine was 41 Ci/mol. Nigericin was a generous gift from Dr. R.L. Hammill (Lilly Research Laboratory, Indianapolis, U.S.A.). Dipropylthiodicarbocyanine was kindly provided by Dr. A.S. Waggoner (Amherst College, Amherst, U.S.A.). Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was given by Dr. P.G. Heytler (Du Pont de Nemours et Cie, Wilmington, U.S.A.). Valinomycin was from Calbiochem and carboxyatractyloside from Boehringer. Bongkrekic acid was prepared according to the method of Limjbach et al. [31], as modified by Lauquin et al. [32]. Catalase C-30 was from Sigma.

Results

Determination of $\Delta\psi$ and ΔpH in particles prepared by sonication under steady state conditions

A typical experiment of the flow dialysis using $S^{14}CN^-$ for $\Delta\psi$ measurement and [^{14}C]methylamine for ΔpH measurement is presented in Fig. 1. In both cases, addition of succinate as oxidizable substrate resulted in a marked uptake of radioactivity which was released upon the subsequent addition of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The difference in radioactivity before and after the addition of the uncoupler was used to calculate $\Delta\psi$ and ΔpH. Upon oxidation of succinate in the presence of oligomycin, the particles developed a $\Delta\psi$ of 95 mV and a ΔpH of 1.5 pH unit corresponding to a proton motive force close to 200 mV (Table I). In the absence of oligomycin, the ΔpH was decreased to a value of about 1 unit. Interconversion of ΔpH and $\Delta\psi$ is suggested by the increase of $\Delta\psi$ found after addition of nigericin (which collapses the ΔpH) and by the increase of ΔpH found after addition of valinomycin (which collapses the $\Delta\psi$). $\Delta\psi$ in particles prepared by sonication was also determined with a SCN^- electrode (not shown), using a mannitol medium (sucrose was found to be inappropriate). $\Delta\psi$ values developed by oxidation of succinate ranged from 110 to 130 mV (see also Kell et al. [18]). $\Delta\psi$ was increased by 50% upon addition of nigericin, probably at the expense of ΔpH.

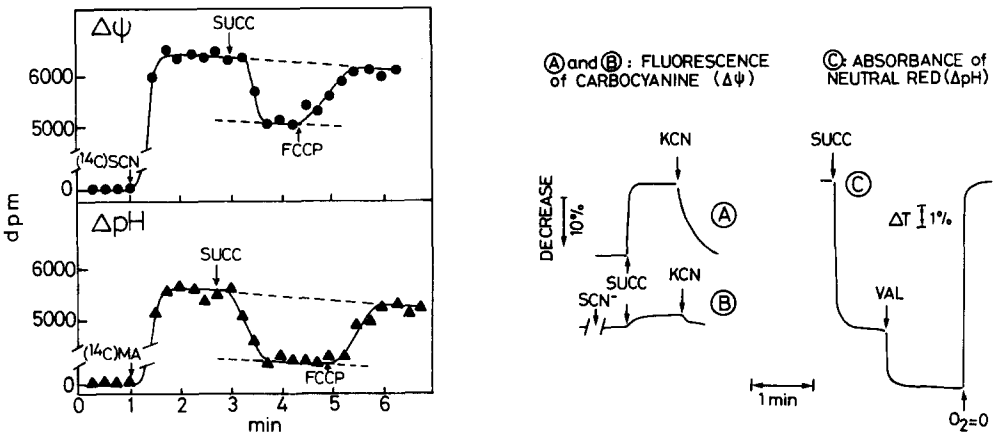


Fig. 1. Measurement by flow dialysis of $\Delta\psi$ and ΔpH in particles prepared by sonication. The reaction medium, placed in the upper chamber of the dialysis cell, contained in a final volume 1.3 ml: 125 mM sucrose, 60 mM KCl, 3 mM Hepes, pH 7.4, 3 μM rotenone, oligomycin 5 μg/mg protein, trace amount of catalase (cf. Methods) and particles prepared by sonication (15 mg protein for the $\Delta\psi$ assay and 20 mg for the ΔpH assay). The same buffered medium as that used in the upper chamber, without oligomycin and catalase, but supplemented with 0.2 M H_2O_2, was pumped through the lower chamber. At time zero, 100 nmol of $S^{14}CN^-$ in 10 μl ($9 \cdot 10^5$ dpm) for measurement or 100 nmol of [^{14}C]methylamine (MA) in 5 μl ($6.3 \cdot 10^5$ dpm) for ΔpH measurement were added to the medium equilibrated at 20°C in the upper chamber. Other details are given in Methods. Additions indicated by arrows correspond to 3 mM succinate and 2 μM FCCP. The effluent was collected every 15 s.

Fig. 2. Generation of $\Delta\psi$ and ΔpH by succinate oxidation in particles prepared by sonication. Monitoring of $\Delta\psi$ by dipropylthiodicarbocyanine fluorescence. Monitoring of ΔpH by neutral red absorbance. The medium used for fluorescence measurement (dipropylthiodicarbocyanine) contained 225 mM sucrose, 30 mM KCl, 1 mM EDTA, 10 mM Hepes, 3 mM succinate, oligomycin 5 μg/mg protein, 3 μM dipropylthiodicarbocyanine and 0.2 mg of particle protein (traces A and B). Final pH 7.4. Final volume 3 ml. Fluorescence measurements were made at 20°C (excitation and emission wavelengths 620 and 670 nm). The medium used for absorbance measurement of neutral red (trace C) contained 120 mM sucrose, 60 mM KCl, 30 mM Hepes, oligomycin 5 μg/ml, 67 μM neutral red and 7.5 mg of particle protein. Final pH 7.4. Final volume 3 ml. The absorbance measurement was made with a dual wavelength spectrophotometer using as wavelength couple 540 and 581 nm. In all cases temperature was 20°C. When present, the following compounds were used at the final concentrations: SCN^- 5 mM, methylamine 5 mM, valinomycin 0.1 μg/mg protein, KCN 1 mM.

TABLE I

DETERMINATION OF $\Delta\Psi$ AND ΔpH LINKED TO SUCCINATE OXIDATION IN SONIC PARTICLES BY THE FLOW DIALYSIS TECHNIQUE

Same conditions as in Fig. 1. The amount of particles prepared by sonication used (prepared as described in Methods) ranged between 15 and 20 mg protein in a final volume of 1.3 ml. Additions were made at the following concentrations: oligomycin 5 μg/mg protein, valinomycin 0.1 μg/mg protein, nigericin 0.2 μg/mg protein, KSCN 5 mM. The $\Delta\Psi$ and ΔpH are calculated from the difference between the two plateau of radioactivity attained after addition of succinate and of FCCP respectively (see Fig. 1). Numbers into parentheses refer to the number of determinations.

Additions	$\Delta\Psi$	ΔpH
Oligomycin	95 ± 4 mV (5)	90 ± 4 mV (2)
Oligomycin + nigericin	increase ≈20%	<20 mV *
Oligomycin + valinomycin	<20 mV * (2)	small increase ≈ 10% (2)
Oligomycin + KSCN	<20 mV *	small increase ≈ 10%
None	no significant change	decrease ≈ 30%

* The value of 20 mV represents the limit below which ΔpH and $\Delta\Psi$ values cannot be determined with accuracy under our experimental conditions.

Fluorimetric and spectrophotometric monitoring of $\Delta\psi$ and ΔpH in particles prepared by sonication

The following assays carried out with particles prepared by sonication show that the fluorescence of dipropylthiodicarbocyanine responds to different effectors of $\Delta\psi$ as expected for inside-out particles. On addition of succinate, the fluorescence intensity of dipropylthiodicarbocyanine was abruptly increased, an effect which is opposite to that found with intact mitochondria (Fig. 2, trace A). The permeant anion SCN^- abolished the fluorescence response of dipropylthiodicarbocyanine in agreement with its collapsing effect on $\Delta\psi$ (Fig. 2, trace B).

The response of neutral red to ΔpH in particles prepared by sonication is illustrated in Fig. 2, trace C. Since neutral red is a penetrant reagent, its specific use as a pH indicator of the internal space of particles requires that the outer phase be strongly buffered. Furthermore to avoid stacking of neutral red molecules, the incubation has to be performed at a pH higher than7 [33]. The medium was therefore buffered with 30 mM hydroxyethylpiperazine ethanesulfonic acid, pH 7.4. Addition of succinate brought about an abrupt decrease absorbance reflecting a rapid internal acidification of the particles. This was followed by a plateau. A subsequent addition of valinomycin resulted in another acid pH shift inside the particles. When anaerobiosis was attained by O_2 exhaustion, the internal pH returned to its initial value. The foregoing assays, in agreement with those based on the flow dialysis technique, indicate partial interconversion of $\Delta\psi$ and ΔpH. The well-known pH indicator, 9-aminoacridine, was found to be not suitable because it responded too slowly to the pH changes induced by succinate oxidation.

ATP-induced changes of dipropylthiodicarbocyanine fluorescence in oligomycin-treated particles

In the absence of oligomycin, addition of ATP to sonic particles loaded with ADP resulted in an increase of dipropylthiodicarbocyanine fluorescence, reflecting the development of a $\Delta\psi$ positive inside. The ATP-induced increase of fluorescence was reversed by oligomycin (Fig. 3, trace A). All further experiments were performed with particles pretreated with oligomycin to prevent ATP hydrolysis.

The fluorescent assays with the carbocyanine dye in the presence of oligomycin were used essentially to detect whether the uptake of ATP in exchange for ADP by ADP-loaded particles could result in a net transport of negative charges inside the particles. Upon addition of ATP to the oligomycin-treated particles, there was a rapid transitory decrease in fluorescence, followed by a slow rise to a steady level (Fig. 3, trace B). Subsequent addition of ADP induced another transitory change of fluorescence opposite to that obtained with ATP. These data parallel those obtained by Laris et al. with intact mitochondria [34]. Bongkrekic acid, a powerful inhibitor of ADP/ATP transport in particles prepared by sonication, inhibited the response of carbocyanine fluorescence to the addition of ATP (Fig. 3, trace C). In contrast, carboxyatractyloside had no effect (Fig. 3, trace D) in agreement with the fact that carboxyatractyloside does not inhibit ADP/ATP transport when added externally to particles prepared by sonication [2]. The imido and methylene analo-

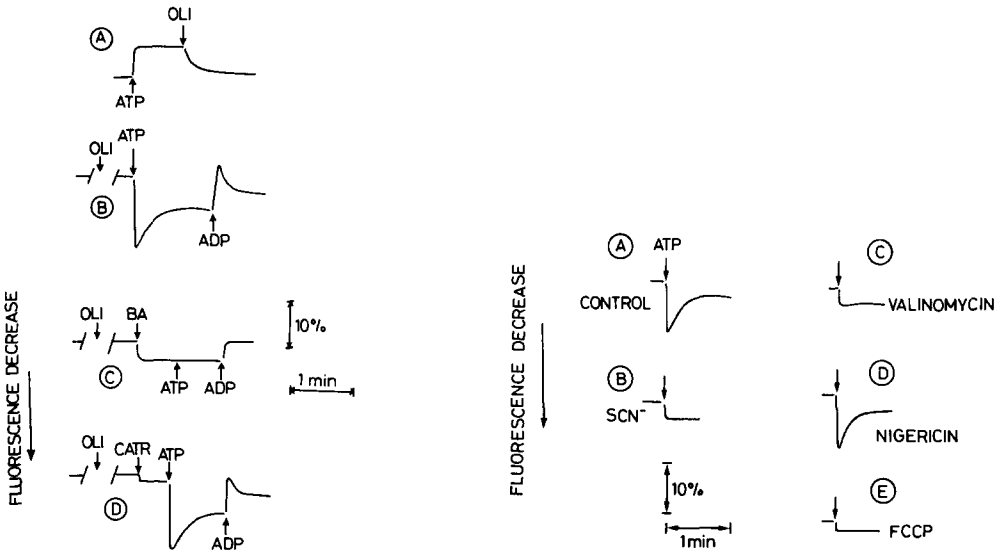


Fig. 3. ATP- and ADP-dependent changes of dipropylthiodicarbocyanine fluorescence in particles prepared by sonication. Effect of inhibitors of ADP/ATP transport. The medium contained 225 mM sucrose, 30 mM KCl, 1 mM EDTA, 3 μM rotenone and 20 mM Hepes buffer pH 7.4. Final volume 3 ml. Particles prepared by sonication (0.2 mg protein) were left in contact with the medium at 20°C for 2 min before addition of ATP. In all assays except in A, oligomycin 5 μg/mg protein was added together with particles prior to ATP. ATP and ADP were added at final concentrations of 100 μM and 600 μM respectively. Other additions were bongkrekic acid (BA) 20 μM and carboxyatractyloside (CATR) 20 μM.

Fig. 4. Modification of the ATP-dependent fluorescence quenching of dipropylthiodicarbocyanine upon changes of $\Delta\psi$ and ΔpH in oligomycin-treated particles. Same experimental conditions as in Fig. 3. The particles (0.2 mg protein) were pretreated with oligomycin for 1 min, and then SCN^- (trace B), valinomycin (trace C), nigericin (trace D), and FCCP (trace E) were added, followed after one min by addition of ATP as indicated by the arrow. Final concentrations were as follows: SCN^- 5 mM, valinomycin 0.1 μg/mg protein, nigericin 0.2 μg/mg protein.

gues of ATP have been tested and compared to ATP for their ability to induce a change of fluorescence of dipropylthiodicarbocyanine in the presence of oligomycin. Whereas a positive response was obtained with intact mitochondria for the above analogues, consistent with their ability to be transported in mitochondria [35,36], virtually no effect was found with particles prepared by sonication. This is in line with the fact that the specificity of sonic particles for ATP(or ADP) is more strict than that of intact mitochondria [2,4].

The size of the fluorescence signal induced by ATP was dependent on protein concentration. At 3 μM dipropylthiodicarbocyanine, it was maximal with 0.05—0.10 mg protein/ml. Replacement of K^+ by Na^+ did not change the size of the signal. On the other hand the signal was markedly diminished in a medium devoid of K^+ or Na^+. There was no significant pH dependence between pH 6.5 and 8.

The ATP-dependent quenching of carbocyanine fluorescence was abolished by the permeant anion SCN^-, by valinomycin in the presence of K^+ and by the uncoupler carbonylcyanide trifluoromethoxyphenylhydrazone. The proton ionophore nigericin had no effect (Fig. 4).

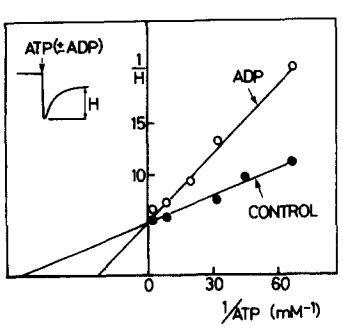
All foregoing assays were carried out at 20°C. When the temperature was

lowered, the response of the carbocyanine fluorescence was slowed down. The half time values for the transitory quenching of fluorescence was less than 1 s at 20°C, 3 s at 10°C and about 10 s at 6°C. The pronounced temperature-dependence of the fluorescence signal is consistent with the high Q_{10} value found for ADP/ATP transport in mitochondria and also in submitochondrial particles prepared by sonication [2—4].

The carbocyanine fluorescence quenching induced by ATP increased with the ATP concentration to reach an apparent saturation value, and conversely, it was decreased by ADP in a competitive manner (Fig. 5). The half maximum effect was attained with an ATP concentration of about 20 μM; the K_i for ADP had roughly the same value.

ATP-induced changes of neutral red absorption in oligomycin-treated particles prepared by sonication

In the following experiment, neutral red was used as an indicator of the internal pH to determined whether the $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange in particles prepared by sonication is charge-compensated by H^+ uptake according to the following equilibrium: $ATP^{4-}_{ex} + H^+_{ex} \rightleftharpoons ADP^{3-}_{in}$. The particles were pretreated with oligomycin, as in the experiments carried out with the carbocyanine dye. The assays were conducted in a sucrose medium in the absence or in the presence of K^+. With a sucrose medium in the absence of K^+, addition of ATP resulted in a transient acidification of the internal phase of the particles (Fig. 6, trace A). On the contrary, a subsequent addition of ADP resulted in a decreased acidification (not shown). Preincubation with bongkrekic acid



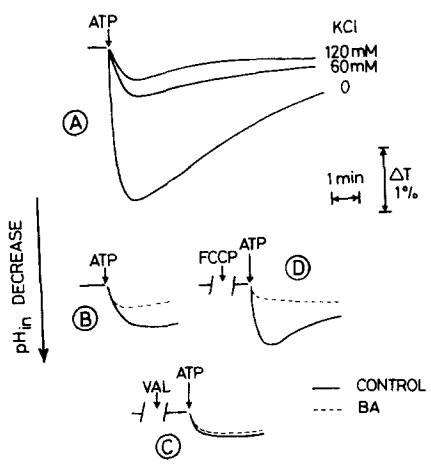


Fig. 5. Effect of ATP concentration on the fluorescence quenching of dipropylthiodicarboxyanine fluorescence in oligomycin-treated particles. Antagonistic effect of ADP. Same experimental conditions as in Fig. 3. When present, ADP was added together with ATP.

Fig. 6. ATP-dependent changes of neutral red absorbance in oligomycin-treated particles. The medium contained 120 mM sucrose, 1 mM EDTA, 30 mM Hepes buffer pH 7.4, 3 μM rotenone, oligomycin 5 μg/mg protein. KCl at the indicated concentrations in trace A and at 60 mM in traces B, C and D. Final volume 3 ml. Particles prepared by sonication (7.5 mg protein) were left in contact with the medium at 20°C for 2 min before addition of ATP (100 μM). When present, bongkrekic acid (BA) was added at a final concentration of 20 μM.

resulted in a substantial decrease of the response to ATP (Fig. 6, trace B). The ATP-induced acidification of the particles was markedly diminished by inclusion of a potassium salt in the medium and totally abolished by addition of valinomycin in the presence of K^+ (Fig. 6, trace C). This observation is reminiscent of recent data by LaNoue et al. [11] on intact mitochondria, showing, by an independent approach, that in the presence of valinomycin and K^+, no H^+ are transported with ATP in ATP_{ex}/ADP_{in} exchange. On the other hand, carbonylcyanide *p*-trifluoromethoxylphenylhydrazone increased both the speed and the size of the neutral red signal on the addition of ATP (Fig. 6, trace D) which is consistent with the facilitation of H^+ movement by uncouplers. It is noteworthy that neutral red responds more slowly to ATP than dipropylthiodicarbocyanine in spite of the fact that both dyes respond with the same velocity to succinate (see Fig. 2). At 20°C, the height of the fluorescence signal of dipropylthiodicarbocyanine induced by ATP was attained in less than 2 s and the return to a steady level in about 30 s; on the other hand the response of neutral red reached its conpletion in about 30 s. H^+ movement monitored by neutral red could therefore be secondary to the charge movement reported by dipropylthiodicarbocyanine.

Effect of SCN^- and nigericin on $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange in respiring particles

Assuming that $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange in particles prepared by sonication is electrogenic, one can predict that the kinetics of this exchange will depend on $\Delta\psi$. For example starting from respiring particles which are able to develop a large proton motive force, a specific decrease in $\Delta\psi$ would result in a decreased activity of ATP_{ex}/ADP_{in} exchange. On the contrary a decrease in ΔpH will not affect the transport activity. Following this reasoning, we studied the effect of SCN^- on ATP_{ex}/ADP_{in} exchange in [^{14}C]ADP-loaded particles. [^{14}C]ADP and [^{14}C]ATP in the loaded particles were in a ratio of 9 to 1. The particles were preincubated with succinate in the presence of oligomycin to develop maximal $\Delta\psi$ and ΔpH. The transport was started by addition of ATP. The released ^{14}C radioactivity sensitive to inhibition by bongkrekic acid, (taken into account to calculate the rate of transport), was present as [^{14}C]ADP to an extent of 90%. Addition of SCN^- at a concentration of 5 mM, sufficient to collapse the $\Delta\psi$ without noticeable secondary effects, resulted in a five-fold decrease of the affinity for ATP_{ex} and in a 30% decrease of the maximal velocity of the exchange (Fig. 7A). In contrast to SCN^-, nigericin and methylamine which collapse the ΔpH had virtually no effect on ATP_{ex}/ADP_{in} exchange (not shown). When ADP was added instead of ATP to the [^{14}C]ADP-loaded particles, the ADP_{ex}/ADP_{in} exchange which ensued was not severely affected by SCN^- (Fig. 7B).

Effect of SCN^- on the ATP_{ex}/ADP_{in} exchange in non-respiring particles prepared by sonication

In this series of experiments, [^{14}C]ADP-loaded particles prepared by sonication were pretreated with rotenone and antimycin to inhibit any residual oxidation of endogenous substrates and by oligomycin to inhibit ATP hydrolysis. In a preliminary assay (Fig. 8), monitoring $\Delta\psi$ in these non-repiring particles by dipropylthiodicarbocyanine indicated that addition of KSCN at a concen-

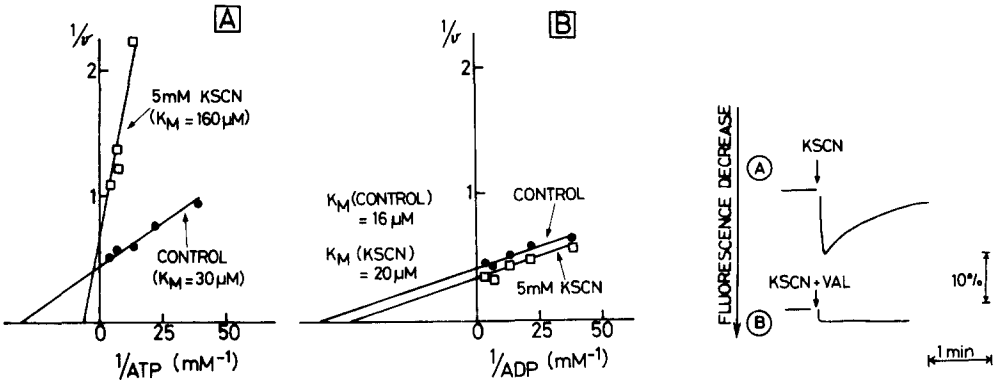


Fig. 7. Effect of SCN^- on kinetics of $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange and $ADP^{4-}_{ex}/ADP^{3-}_{in}$ exchange in respiring particles prepared by sonication. The medium consisted of 225 mM sucrose, 30 mM KCl, 1 mM EDTA, 10 mM Mops, 5 mM succinate, 1 μM rotenone, and oligomycin 5 μg/mg protein. Final pH 6.5. Final volume 1 ml. Temperature 6°C. The [^{14}C]ADP-loaded particles (1 mg protein) were preincubated in the above medium, in the presence or absence of KSCN, for two min prior addition of ATP. The ATP_{ex}/ADP_{in} exchange initiated by addition of ATP was stopped after 20 s and 40 s by 20 μM bongkrekic acid. After high speed centrifugation, the supernatants were recovered and their radioactivity determined by liquid scintillation. A control was performed for the spontaneous release of ^{14}C radioactivity, insensitive to bongkrekic acid [2].

Fig. 8. Effect of a high concentration of KSCN on the fluorescence of dipropylthiodicarbocyanine in oligomycin-treated non-respiring particles prepared by sonication. The medium consisted of 230 mM sucrose, 1 mM EDTA, 5 mM Mops, 1 μM rotenone, oligomycin 5 μg/mg protein and 3.3 μM dipropylthiodicarbocyanine. Final pH 6.5. Final volume 3 ml. Temperature 20°C. The amount of particles was 0.2 mg protein. KSCN was added at the final concentration of 30 mM (arrows in Traces A and B). When present, valinomycin was added at the concentration of 0.2 μg/mg protein together with KSCN (Trace B).

tration of 30 mM generated a transient diffusion potential negative inside which vanished in less than 30 s. This diffusion potential due to the entry of SCN^- was abolished when valinomycin was added together with KSCN. Results of a kinetic assay performed to test the effect of a transient difffusion potential

TABLE II

EFFECT OF SCN^- ON $ATP^{4-}_{ex}/ADP^{3-}_{in}$ EXCHANGE IN NON-RESPIRING PARTICLES PREPARED BY SONICATION

The medium consisted of 230 mM sucrose, 1 mM EDTA, 5 mM Mops, 1 μM rotenone, and oligomycin 5 μg/mg protein. Final pH 6.5, final volume 1 ml. Temperature: Expt. 1 : 20°C, Expt. 2 : 10°C. The [^{14}C]ADP-loaded particles (1 mg protein) were left for 4 min in the medium prior addition of ATP. KSCN was added at final concentration of 30 mM and valinomycin at 0.1 μg/mg protein. Incubation time following addition of ATP 20 s. Other conditions as in Fig. 7.

Addition order	% exchange *	
	Expt. 1	Expt. 2
KSCN added 4 min before ATP	85	37
KSCN added together with ATP	44	18
KSCN plus valinomycin added 4 min before ATP	72	30
KSCN plus valinomycin added together with ATP	70	40

* Percentage of exchange rather than rates were preferred to express the data because kinetics was not resolved in this experiment with enough accuracy at the temperature used.

induced by SCN^- uptake in the ATP_{ex}/ADP_{in} exchange in non-respiring particles are presented in Table II. When ATP was added together with KSCN, the rate of ATP transport was markedly decreased as compared to a control assay in which ATP was added four min after KSCN addition. (Table II, lines 1 and 2). That the adverse effect of KSCN is actually related to a change in $\Delta\psi$ is substantiated by the absence of inhibitory effect of KSCN or even a slight stimulation when the medium is supplemented with valinomycin (Table II, lines 3 and 4). In that case, negative charges brought about by penetration of SCN^- are compensated by the valinomycin-induced entry of K^+.

Discussion

The purpose of the present study was two fold, (1) to determine whether particles prepared by sonication loaded with ADP and $MgCl_2$ and competent in ADP/ATP transport are able develop a $\Delta\psi$ and(or) a ΔpH of significant value, (2) to determine whether the $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange that they catalyse responds to changes of either $\Delta\psi$ or ΔpH and thus is electrogenic or electroneutral.

1. $\Delta\psi$ and ΔpH in sonic $MgCl_2$ particles

Respiring sonic $MgCl_2$ particles are able to develop a $\Delta\psi$ (positive inside) and a ΔpH (acidic inside) with values as high as 90—95 mV, as assessed by the flow dialysis technique. On the other hand, they are competent in ADP/ATP transport. They are therefore a suitable material to determine whether the $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange is accompanied by a transport of charges (electrogenic transport) or is charge-compensated (electroneutral transport).

2. The electrogenic nature of ADP/ATP transport

At neutral pH, ATP bears 4 negative charges and ADP 3 negative charges. The question as to whether the ATP^{4-}/ADP^{3-} exchange in isolated mitochondria is electrogenic has been largely debated. A control of ADP/ATP exchange in intact mitochondria by a $\Delta\psi$ positive outside and negative inside was postulated by Klingenberg et al. [6] to explain the finding that ATP is taken up into tightly coupled mitochondria at a lower rate than ADP and that the rate of ADP and ATP uptakes become equal upon addition of an uncoupler. Souverijn et al. [37] showed however that it is essentially the affinity for ATP which is low in well coupled mitochondria, the maximal velocity being not significantly modified.

More convincing evidence for the electrogenicity of ADP/ATP transport stems from recent data based on $\Delta\psi$ determinations, showing that there is a relationship between $\Delta\psi$ values and the distribution of ATP and ADP inside and outside the mitochondria [7]. H^+ and cation movements, possibly associated with ADP/ATP transport in mitochondria, have also been measured by Wulf et al. [10] in the presence of oligomycin and inhibitors of electron transport in order to exclude some other types of H^+ movement. The ADP/ATP exchange was found to be both partially electrogenic and partially electroneutral. Electroneutrality calculated from the ratio of H^+ taken up to ATP added was approximated to 38% in ATP_{ex}/ADP_{in} exchange. On the other

hand, ADP_{ex}/ATP_{in} exchange was found to be about 50% electroneutral and 50% electrogenic. However, these data should be accepted with some reservation because H^+ uptake was referred to the added ATP or ADP and not to the transported ATP or ADP. A recent reappraisal by LaNoue et al. [9,11] showed that H^+ movement is not an inherent part of the ADP/ATP carrier mechanism; H^+ would move in a passive manner to balance the charge separation brought about by the activity of a fully electric ADP/ATP carrier.

A different approach by Laris [34], based on the use of dipropylthiodicarbocyanine, a selective probe of $\Delta\psi$, indicated that $\Delta\psi$ in intact mitochondria responds to added ATP or ADP in a way which is sensitive to specific inhibitors of ADP/ATP transport. In this work, however, no mention was made about the effect of ΔpH on ATP/ADP transport. The finding by Shertzer et al. [8] that the exchange of ATP_{in} for ADP_{ex} in reconstituted phospholipid vesicles is stimulated by various agents that collapse the $\Delta\psi$ also points to the electrogenicity of ADP/ATP transport. All the above-mentioned data were obtained with intact mitochondria. Although agreement was reached to conclude that ADP/ATP exchange in mitochondria is at least partially electrogenic, the direct coupling of a H^+ movement to the ADP carrier, thus confirming some electroneutrality to the exchange, was not clearly assessed, except in ref. 11.

The work described here represents the first attempt to assess the electrical properties of ADP/ATP transport in inside-out submitochondrial particles. There are at least two ways to determine whether $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange in oligomycin-treated particles is electrogenic or electroneutral: (1) to study the movement of negative charges and H^+ resulting from the uptake of ATP in nonrespiring particles which do not develop a significant $\Delta\psi$ and ΔpH; (2) to study the effect of a change in $\Delta\psi$ and ΔpH on kinetics of $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange. Both aspects were considered in this paper. First, we found that ATP uptake into sonic particles treated with oligomycin results in a transport of negative charges into the internal space of the particles. That this transport of charges is specifically linked to $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange is strongly suggested by the following findings. (1) ATP uptake and ADP uptake result in opposite movements of negative charges which can be monitored by dipropylthiodicarbocyanine, (2) The movement of charges is inhibited by bongkrekic acid, a specific inhibitor of ADP/ATP transport in particles prepared by sonication, (3) Analogs of ADP or ATP, which are not transported in sonic particles, do not give rise to a charge movement, (4) There is a strong temperature dependence for the charge movement as for ADP transport. On the other hand, assays carried out with neutral red showed that ATP uptake in exchange for ADP efflux is accompanied by a H^+ movement sensitive to bongkrekic acid. However this H^+ movement is maximal when the incubation medium is devoid of KCl. It is decreased in the presence of K^+, and totally abolished when valinomycin is present together with K^+. On the contrary, it is enhanced by addition of the uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. This situation suggests that H^+ move independently from ATP and are not transported by the ADP/ATP carrier. Indeed, comparison of H^+ movement monitored by neutral red and movement of negative charges monitored by dipropylthiodicarbocyanine show that H^+ move more slowly than the negative charges carried by ATP^{4-}. It is therefore possible that H^+ are passively transported

across the membrane following ATP transport, essentially to neutralize the excess of negative charges resulting from $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange.

Another evidence of the electrogenic nature of ATP/ADP exchange in sonic particles is based on the effect of $\Delta\psi$ changes on kinetics of transport. The $\Delta\psi$ was modified in two ways. In respiring particles, the $\Delta\psi$, positive inside, developed by respiration was collapsed by addition of a small concentration KSCN (5 mM) whereas in non-respiring particles, a transient diffusion potential, negative inside, was generated by addition of a large concentration of KSCN (30 mM). In the case of respiring particles, one can predict that an electrogenic $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange will be favoured by a $\Delta\psi$ positive inside. This is in fact consistent with the decrease of the rate of ATP/ADP exchange when the $\Delta\psi$ is collapsed by SCN^-. On the other hand, there is no evidence for an electroneutral ATP/ADP exchange since the exchange is not modified when the ΔpH is collapsed by nigericin. In non-respiring particles, the fact that the transient $\Delta\psi$ generated by the entry of a large amount of SCN^- counteracts the $ATP^{4-}_{ex}/ADP^{3-}_{in}$ exchange is also in agreement with the electrogenic nature of this exchange. A deleterious effect of KSCN per se on the ATP/ADP carrier is unlikely since the inhibition caused by KSCN is reversed by valinomycin, which facilitates the entry of K^+ and thereby the neutralization of negative charges carried by SCN^-. The above data indicate a clear relationship between the $\Delta\psi$ of the mitochondrial membrane and the kinetics of ADP/ATP transport. It remains to be explained why the generation of $\Delta\psi$, created by the energization of the mitochondrial membrane, modifies more the affinity of the adenine nucleotide carrier, particularly for ATP, than the rate of transport. An indirect effect of $\Delta\psi$ on the conformation of the carrier cannot be excluded.

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