ANILINONAPHTHALENE SULFONATE AND OTHER SYNTHETIC IONS AS MITOCHONDRIAL MEMBRANE PENETRANTS: AN H^+ PULSE TECHNIQUE STUDY

A.A. JASAITIS, LA VAN CHU and V.P. SKULACHEV
Department of Bioenergetics, Laboratory of Bioorganic Chemistry, Moscow State University, Moscow, USSR

Received 7 February 1973

1. Introduction

The study of the energy-transducing mechanisms of mitochondrial membrane has revealed the existence of a system carrying out ion uptake and extrusion when the membrane is energized. This system, being non-specific to details of the ion structure (many synthetic ions could be involved) proved to be very specific to the charge on the molecule: cations are taken up while anions are extruded from mitochondria under energization [1–5]. Similar behaviour was found with two fluorescent dyes: anilinonaphthalene sulfonate (ANS^-) and auramine O. The former, an anion, was released into the incubation medium, the latter, a cation, was absorbed by energized mitochondria. Energization did not influence the distribution of the uncharged ANS amide [6].

The charge-specific ion responses of mitochondria have been explained [4, 5] as being a result of electrophoretic transport of ions through the membrane down the electric potential gradient generated, according to Mitchell [7, 8], by respiration or ATP hydrolysis.

An alternative explanation was put forward by Azzi [6] who suggested that energization changes the surface charge of the membrane which results in a change in the binding of ions on the membrane surface.

The former point of view was supported by a study on artificial phospholipid membrane, showing that any ion penetrating through this membrane can be involved in the energy-dependent charge-specific response of mitochondria [2, 9–12]. However, some ions affecting mitochondria (e.g. ANS^-), proved inactive with artificial membranes if some special treatments were not employed [10]. Therefore, it was desirable to study whether such ions are mitochondrial membrane penetrants. The present paper summarizes the results of an investigation along these lines.

It has been found that anilinonaphthalene sulfonate and phenyl dicarbaundecaborane anions, *N*, *N*-dibenzyl, *N*, *N*-dimethyl ammonium and tetrabutyl ammonium cations, like SCN^- or K^+ (+ valinomycin), induce an increase in the rate of titration of the intramitochondrial buffer by the external H^+ ions, the effect being greatly potentiated by a protonophorous uncoupler. In the presence of an uncoupler, the rate of titration of the intramitochondrial buffer tends to infinity at the infinite concentration of the above ions. These data suggest that the charged form of anilinonaphthalene sulfonate and other synthetic ions studied readily penetrate through the inner mitochondrial membrane.

2. Materials and methods

To determine the penetrating ability of the ion studied, the H^+ pulse technique was employed [13, 14]. Small amounts of HCl were added to mitochondria whose permeability to H^+ ions had been increased by a protonophorous uncoupler, *p*-trifluoromethoxycarbonylcyanidephenylhydrazone (FCCP). In such conditions, the rate of the movement of H^+ ions into mito-

Abbreviations:
ANS^-, Anilinonaphthalene sulfonate anion; DDA^+, *N*, *N*-dibenzyl, *N*, *N*-dimethyl ammonium cation; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone; TBA^+, tetrabutyl ammonium cation; PCB^-, phenyl dicarbaundecaborane anion.

North-Holland Publishing Company – Amsterdam

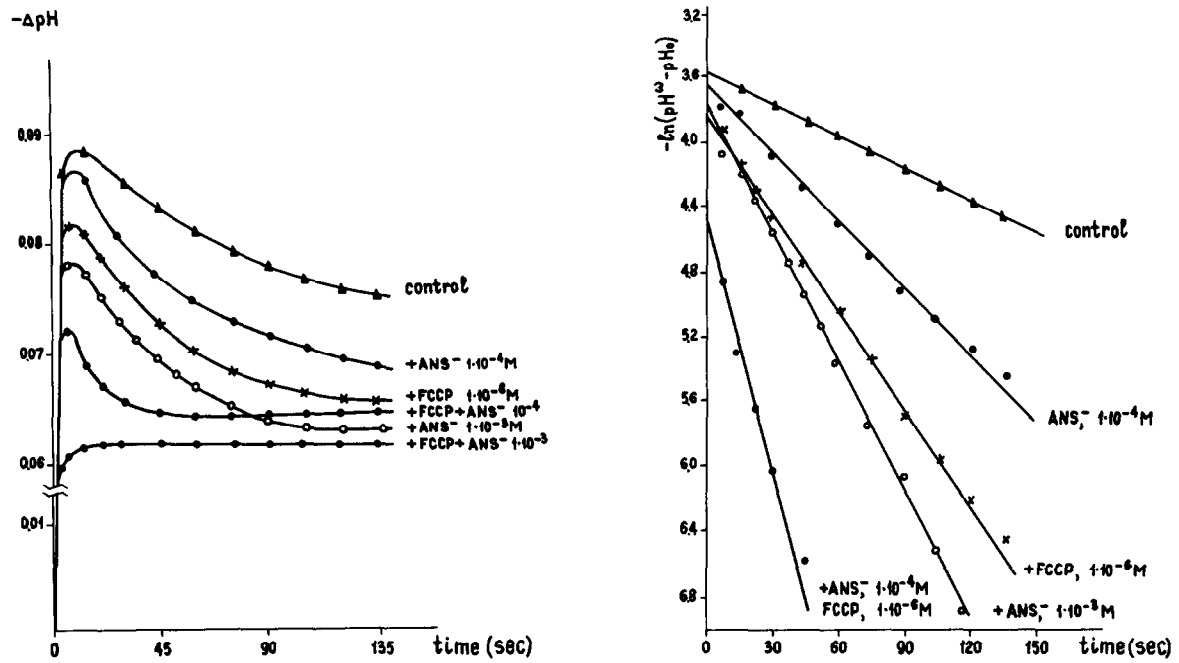


Fig. 1. The effect of ANS^- on the pH changes induced by adding HCl to anaerobic rat liver mitochondria. Incubation mixture (see Materials and methods) contained 5.7 mg protein/ml.

chondria down the H^+ concentration gradient was limited by formation of the H^+ diffusion potential (positive inside the mitochondrion). Discharging the membrane by the influx of a penetrating anion or the efflux of a penetrating cation should greatly decrease the time required for the added H^+ ions to penetrate into the mitochondrial matrix, the result being an increase in the rate of titration of the intramitochondrial buffer capacity. So, the penetrating ability of different ions can be compared by the half-times of the intramitochondrial buffer titration.

Rat liver mitochondria were prepared as described earlier [3]. For homogenizing the tissue and washing the mitochondria, a solution of 0.3 M sucrose and 1 mM Tris-HCl buffer was used. The pellet of washed mitochondria was suspended in 0.3 M sucrose and stored at 0°. In the H^+ pulse experiments, mitochondria were incubated for 10–15 min at room temp. in the solution containing 0.125 M KCl, 0.03 M sucrose, 1×10^{-6} M rotenone, mitochondria (6–9 mg protein/ml) and the additions indicated below. Before the experiment, the reaction mixture was saturated with argon. The pH changes induced by addition of 5×10^{-3} M HCl were monitored with a pH recorder.

3. Results and discussion

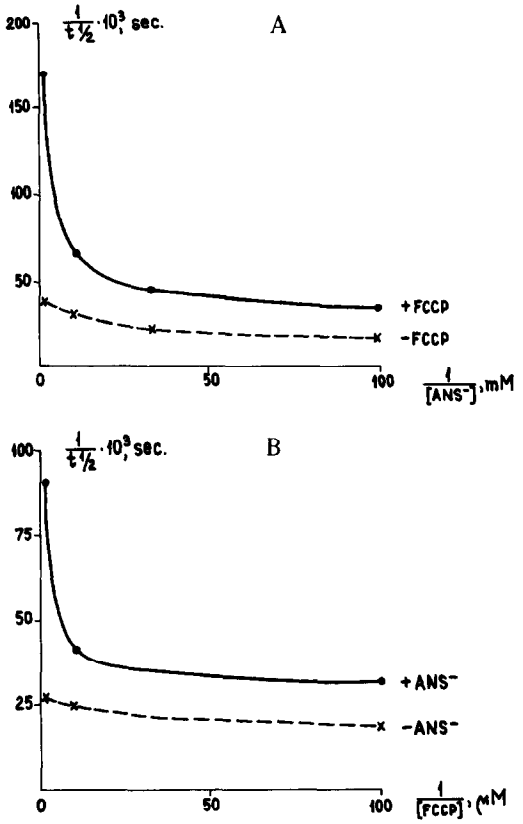
The results of a typical experiment are shown in fig. 1. It is seen (fig. 1A) that addition of HCl to an anaerobic suspension of mitochondria results in acidification of the mixture followed by a decay of the pH change (titration of intramitochondrial buffer). The decay rate increased when the mixture was supplemented with FCCP. Addition of ANS^- also resulted in some increase in the decay rate. Combination of FCCP and ANS^- is the most effective. At concentrations of ANS^- and FCCP of 1×10^{-3} M and 1×10^{-6} M, respectively, the rate of titration of the intramitochondrial buffer proves to be too high to be measured by the technique used. Fig. 1B shows the time course of values of $-\ln(pH^{\omega} - pH_0)$, where pH_0 is the pH level outside mitochondria at the moment of measurement, and pH^{ω} the pH level outside mitochondria after the added H^+ ions had equilibrated the intramitochondrial buffer. From this figure, the time of the H^+

Table 1

The time of half-equilibration of H^+ ions across the mitochondrial membrane ($t_{1/2}$) in the presence of different ionized compounds.

Exp.	Concentration of mitochondrial protein (mg/ml)	Additions (M)	$t_{1/2}$ (sec)
1.	5.7	–	95
		FCCP (1×10^{-6})	47
		ANS^- (1×10^{-3})	35
		FCCP (1×10^{-6}) + ANS^-(1×10^{-3})	<7
		TBA^+(2.5×10^{-2})	37
		FCCP (10^{-6}) + TBA^+(2.5×10^{-2})	7
		Triton X-100 (0.1%)	<7
2.	5.5	–	102
		FCCP (1×10^{-6})	33
		ANS^-(1×10^{-4})	48
		FCCP (1×10^{-6}) + ANS^-(1×10^{-4})	12
3.	8.8	–	72
		FCCP (1×10^{-6})	51
		PCB^-(1×10^{-4})	44
		FCCP (1×10^{-6}) + PCB^-(1×10^{-4})	18
4.	9.0	–	43
		FCCP (1×10^{-6})	33
		DDA^+(2×10^{-2})	28
		FCCP (1×10^{-6}) + DDA^+(2×10^{-2})	<7
		Valinomycin (7×10^{-8})	42
		FCCP (1×10^{-6}) + valinomycin (7×10^{-8})	<7
5.	7.5	–	98
		FCCP (1×10^{-6})	42
		SCN^-(1×10^{-2})	28
		FCCP (1×10^{-6}) + SCN^-(5×10^{-3})	21
		FCCP (1×10^{-6}) + SCN (1×10^{-2})	<7
6.	8.0	–	75
		FCCP (1×10^{-6})	56
		ANS^-(10^{-3})	28
		FCCP (1×10^{-6}) + ANS^- (10^{-3})	<7

In exp. 6 the incubation mixture (see text) contained 0.025 M choline bromide instead 0.125 KCl; sucrose concentration was increased to 0.2 M.



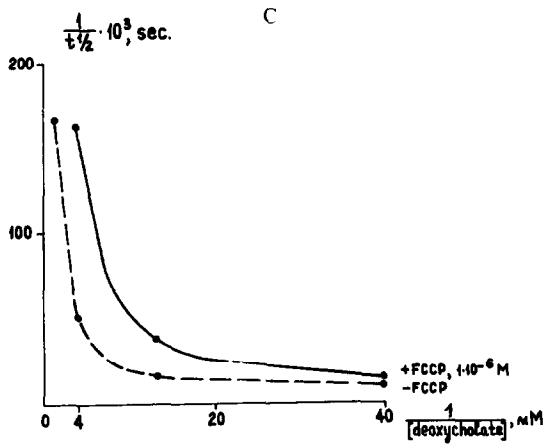


Fig. 2. The concentration dependences of the ANS^- and FCCP effects on the time of half-equilibration of H^+ ions across the mitochondrial membrane. Incubation mixture (see text) contained 8.2 (exp. A), 9.3 (exp. B) and 10.5 (exp. C) mg protein/ml. In exp. A and C, solid curves, all samples were supplemented with 1×10^{-6} M FCCP, in exp. B, solid curve, with 2×10^{-4} M ANS^-.

half-equilibrium across the mitochondrial membrane ($t_{1/2}$) can be obtained (see [13]). This criterion was employed in the following experiments whose results are summarized in table 1.

It is seen that ANS^-, phenyl dicarbaundecaborane anion (PCB^-), dibenzyl dimethyl ammonium cation (DDA^+) and tetrabutyl ammonium cation (TBA^+) greatly decrease the $t_{1/2}$ value. In table 1 the effectivities of these synthetic compounds are compared with those of K^+ (+ valinomycin) and SCN^-, studied earlier by Mitchell and others [13, 15]. One can see, for example, that in the presence of FCCP, 1×10^{-4} M ANS^- proves to be somewhat more effective than 5×10^{-3} M SCN^- (cf. exp. 2 and 5). Substitution of KCl in the incubation mixture by choline bromide is without effect on the ANS^- action (exp. 6). In fig. 2A and B values $1/t_{1/2}$ are plotted against 1/[ANS] or 1/[FCCP], respectively. It is seen that effects of FCCP and ANS^- on the H^+ equilibrium are not additive: the FCCP action is greatly potentiated by ANS^- and *vice versa*. This means that ANS^-, like SCN^-, does not induce the H^+ permeability which should increase if ANS^- acted as protonophore or detergent. Similar data were obtained when the effect of other synthetic ions was studied. In the same system the anionic detergent, deoxycholate, greatly decreased $t_{1/2}$ even in the absence of FCCP (fig. 2C). Similar data were obtained when the non-ionic detergent, Triton X-100, was used.

The above results show that ANS^-, PCB^-, DDA^+ and TBA^+ like such penetrating ions as K^+ (+ valinomycin) and SCN^-, greatly increase the rate of titration of intramitochondrial buffer when the membrane of mitochondria is made H^+ permeable. This observation indicates that ANS^- and other ions studied are mitochondrial membrane penetrants. Hence, distribution of these ions between the extramitochondrial and matrix spaces should be affected by an electric potential difference across the membrane. Thus, the suggestion is confirmed that by measuring concentrations of ANS^-, PCB^- etc. in the extramitochondrial solution one can follow the formation of the membrane potential in mitochondria and submitochondrial particles [4, 10]. Further, the fact that ANS^- is a mitochondrial membrane penetrant adds to the validity

of the explanation of the energy-dependent ANS^- fluorescence changes as resulting mainly from the redistribution of ANS^- between the incubation medium and mitochondria due to the transmembrane electrophoretic movement of ANS^- [10, 16–18]. As to some other factors which may change the ANS^- fluorescence intensity in mitochondria affecting the quantum yield, their contribution seems to be rather small, according to the last study by Barret-Bee and Radda [19].

Acknowledgement

The authors are grateful to Miss T.I. Kheifets for correcting the English version of the paper.

References

[1] E.A. Liberman, V.P. Topali, L.M. Tsofina, A.A. Jasaitis and V.P. Skulachev, Nature 222 (1969) 1076.
[2] L.L. Grinius, A.A. Jasaitis, J.P. Kadziauskas, E.A. Liberman, V.P. Skulachev, V.P. Topali, L.M. Tsofina and M.A. Vladimirova, Biochim. Biophys. Acta 216 (1970) 1.
[3] L.E. Bakeeva, L.L. Grinius, A.A. Jasaitis, V.V. Kuliene, D.O. Levitsky, E.A. Liberman, I.I. Severina and V.P. Skulachev, Biochim. Biophys. Acta 216 (1970) 13.
[4] E.A. Liberman and V.P. Skulachev, Biochim. Biophys. Acta 216 (1970) 30.
[5] V.P. Skulachev, FEBS Letters 11 (1970) 301.
[6] A. Azzi, Biochem. Biophys. Res. Commun. 37 (1969) 254.
[7] P. Mitchell, Biol. Rev. 41 (1966) 445.
[8] P. Mitchell, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation (Glynn Research, Bodmin, 1966).
[9] E.A. Liberman and V.P. Topali, Biochim. Biophys. Acta 163 (1968) 125.
[10] V.P. Skulachev, Current Topics in Bioenergetics 4 (1971) 127.
[11] V.P. Skulachev, in: Energy transduction in respiration and photosynthesis, eds. E.C. Slater, E. Quagliariello, S. Papa and C.S. Rossi (Adriatica Editrice, Bari, 1971). p. 99.
[12] V.P. Skulachev, in: FEBS Meet. Abstr., 7th, Varna, 1971 p. 56.
[13] P. Mitchell and J. Moyle, Biochem. J. 104 (1967) 588.
[14] P. Mitchell and J. Moyle, in: Biochemistry of Mitochondria, eds. E.C. Slater, Z. Kaniuga and L. Wojtczak (Acad. Press, London, New York, 1967) p. 53.
[15] P. Scholes and P. Mitchell, J. Bioenergetics 1 (1970) 61.
[16] A.A. Jasaitis, V.V. Kuliene and V.P. Skulachev, Biochim. Biophys. Acta 234 (1971) 177.
[17] V.P. Skulachev, J. Bioenergetics 3 (1972) 25.
[18] L.L. Grinius, M.D. Il'ina, E.I. Mileykovskaya, V.P. Skulachev and G.V. Tikhonova, Biochim. Biophys. Acta 283 (1972) 442.
[19] K. Barrett-Bee and G.K. Radda, Biochim. Biophys. Acta 267 (1972) 211.