Mn^{2+} INSIDE SUBMITOCHONDRIAL PARTICLES AS A TOOL FOR STUDYING THE FUNCTIONAL STATE OF THE MITOCHONDRIAL MEMBRANE

E. A. IMEDIDZE, I. E. DROBINSKAYA, T. M. KERIMOV, E. K. RUUGE and I. A. KOZLOV
Department of Bioenergetics, A.N. Belozersky Laboratory of Bioorganic Chemistry and Department of Physics, Moscow State University, Moscow 117234, USSR

Received 21 August 1978
Revised version received 2 October 1978

1. Introduction

The electron spin resonance technique may be used in studying Mn^{2+} transport across the mitochondrial membrane [1–6]. The distribution of Mn^{2+} between the matrix and the extramitochondrial space has been studied [1–5]. Dell' Antone et al. [6] observed the exit of Mn^{2+} from submitochondrial particles in exchange for an H^+ during treatment of the particles with the ionophore A23187.

In this paper it is shown by the ESR method that Mn^{2+} can be in a free state inside the submitochondrial particles, as well as in a complex with protein and (or) lipid membrane components. The drop in pH in the space inside the submitochondrial particles leads to desorption of Mn^{2+} and thus to an increase in the concentration of free Mn^{2+} inside the particles. By monitoring the change in the concentration of free Mn^{2+} inside the particles by the ESR method, it is possible to follow ΔpH generation on the mitochondrial membrane.

2. Materials and methods

The mitochondria were obtained from beef heart according to [7]. Mn-SMP were obtained by the modified method in [8]. Ultrasonic treatment of the mitochondria was carried out in a medium containing: 0.25 M sucrose; 1 mM succinate; 1 mM ATP; 5 mM $MgSO_4$; 50 mM $MnCl_2$; 10 mM HEPES (pH 7.9). The particles were resuspended 4 times in a Cl^--free medium containing: 0.25 M sucrose; 0.2 mM EDTA; 10 mM HEPES–H_2SO_4 (pH 7.5), 1 ml medium to 1 mg particle protein, and centrifuged at 105 000 × *g* for 45 min. The particles thus washed were suspended in a medium containing 0.25 M sucrose, 10 mM HEPES–H_2SO_4 (pH 7.0), 40 mg particle protein/ml, and left overnight at −12°C. The ATPase activity of the particles was 0.5 μmol/min/mg protein (pH 8.3, 25°C). ESR measurements were carried out with Varian E-4 and Thomson CSF-250 spectrometers at 20°C. The membrane potential was measured by the method of penetrating ions [9]. PCB^- was used as a lipid-soluble ion.

Abbreviations: Mn-SMP, submitochondrial particles with a high Mn^{2+} content; ESR, electron spin resonance; ClCCP, *m*-chlorocarbonylcyanide phenylhydrazone; PCB^-, phenyl dicarba undecaborane anion

3. Results and discussion

It is known that free Mn^{2+} in water solution produce a characteristic ESR spectra of 6 lines. The magnitude of the ESR signal is proportional to the free Mn^{2+} concentration. Mn^{2+} binding by negatively-charged ligands causes the magnitude of the ESR signal to decrease [4,5].

Figure 1 shows how the concentration of free Mn^{2+} changes in the Mn-SMP preparation on adding EDTA, which does not penetrate the membrane. The drop in the free Mn^{2+} concentration was traced by the decrease in the magnitude of the ESR signal. From the results obtained it can be seen that ~96% of the free Mn^{2+} in the Mn-SMP preparation can be bound by EDTA

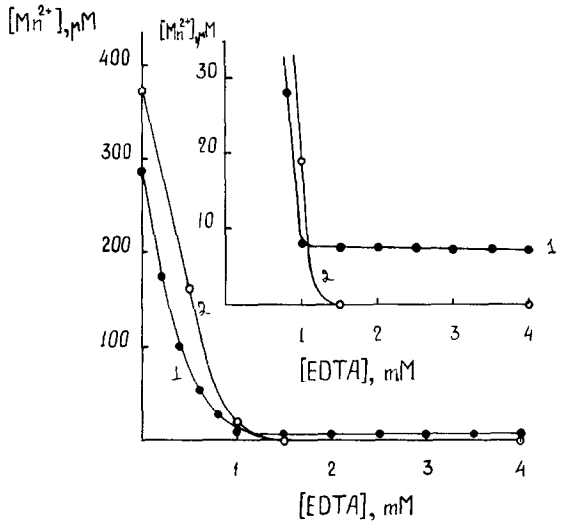

Fig.1. Changes in the concentration of free Mn^{2+} in the Mn-SMP preparation on adding EDTA. The medium contained 0.25 M sucrose, 10 mM HEPES–H_2SO_4, Mn-SMP 10 mg/ml. The final pH was 7.0. Curve 1 was without and curve 2 with A23187 (10 μg/ml). To determine the free Mn^{2+} concentrations, the ESR spectra of Mn-SMP preparation were compared with the ESR spectra of the $MnCl_2$ solutions of known concentrations (insert).

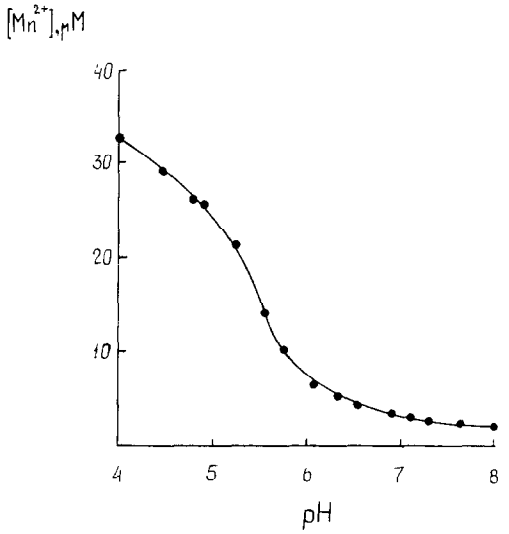

Fig.2. The dependence of the concentration of free Mn^{2+} inside the submitochondrial particles on pH. The medium contained 0.25 M sucrose, 10 mM HEPES, 3 mM EDTA, Mn-SMP 10 mg/ml. The pH changes were effected by small additions of KOH or HCl.

(curve 1). This indicates that either a large part of the free Mn^{2+} is localised on the outside of the submitochondrial particles, or that the Mn^{2+} leaves the particles under the effect of EDTA. Part of the free Mn^{2+} (about 4% in the experiment cited in fig.1; in different Mn-SMP preparations this quantity varied from 0.3–0.7 nmol Mn^{2+}/mg protein) remains inaccessible to EDTA, and is apparently localised in the water phase inside the submitochondrial particles. This conclusion is confirmed by experiments with the ionophore A23187. In the presence of A23187, which sharply raises the penetrability of the mitochondrial membrane to Mn^{2+} [6,10], EDTA binds all the free Mn^{2+} in the Mn-SMP preparation (fig.1, curve 2). Treatment of Mn-SMP with the non-ionic detergent Triton X-100 gives similar results. In the presence of 0.4% Triton X-100, 3 mM EDTA binds all the free Mn^{2+} in the Mn-SMP preparation (not shown).

In subsequent experiments we studied the behaviour of that fraction of the free Mn^{2+} in the Mn-SMP preparation that was not accessible to externally added EDTA. All the ESR measurements in these experiments were carried out in the presence of 3 mM EDTA.

Figure 2 shows that the concentration of free Mn^{2+} inside the submitochondrial particles changes when the pH of the incubation medium is changed*. From the results obtained it can be seen that, at neutral pH, a large part of the Mn^{2+} in the particles is in a bound state, and a drop in pH leads to desorption of Mn^{2+} in the water inside the particles.

The effect of the increase in free Mn^{2+} concentration in the medium within the submitochondrial particles, as a result of the drop in pH, may be used to measure the ATP-dependent generation of ΔpH on the membrane of the particles.

Figure 3 shows the ESR spectra of Mn^{2+} inside the particles in the absence (curve 1) and in the presence (curve 2) of Mg-ATP**. The presence in the incuba-

* Changes in pH from 8.0 to 4.5 in a solution containing 3 mM EDTA and 0.3 mM Mn^{2+} (without submitochondrial particles) do not lead to the appearance of a free Mn^{2+} ESR signal

** In a special experiment it was shown that Mg^{2+} in a concentration of 1-10 mM is not capable of competing with Mn^{2+} for complex formation with EDTA

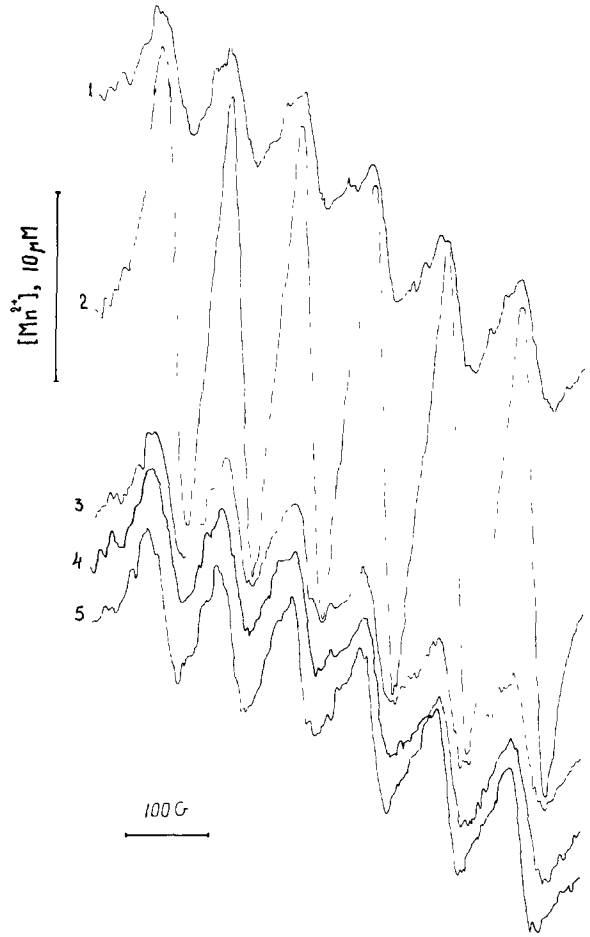

Fig.3. ESR spectra of the Mn^{2+} inside the submitochondrial particles. The medium contained 25 mM KCl, 0.25 M sucrose, 10 mM HEPES–H_2SO_4, pH 7.0, 3 mM EDTA, 10 mM $MgSO_4$, Mn-SMP 10 mg/ml and different additions. Curve 1, none; curve 2, 5 mM ATP; curve 3, 5 mM ATP, 50 mM NH_4Cl; curve 4, 5 mM ATP, 2 μM ClCCP; curve 5, 5 mM ATP; oligomycin 10 μg/ml. ATP additions were made immediately before measurements were made. The time of the spectrum recording was 5–10 min.

tion medium of Cl^-, which penetrates the membrane [11], should ensure the ATP-dependent generation of ΔpH on the particles' membrane [12]. Indeed, as can be seen from the result obtained, the ATP hydrolysis is accompanied by a sharp increase in the free Mn^{2+} concentration in the particles.

Addition to the incubation medium of NH_4Cl, which eliminates the formation of ΔpH on the membrane, prevents the ATP-induced increase in the concentration of free Mn^{2+} in the intraparticle medium (fig.3, curve 3). Addition of ClCCP, the uncoupler of oxidative phosphorylation, which causes the complete de-energisation of the membrane (ΔpH = 0), leads to a similar result (fig.3, curve 4). Finally, the observed effect of the increase in the free concentration of Mn^{2+} inside the particles is blocked by oligomycin, the inhibitor of mitochondrial ATPase (fig.3, curve 5). Figure 4 shows the third line of the ESR spectra of Mn^{2+} inside the Mn-SMP preparation in the presence of ATP and various anions, which are able to penetrate the mitochondrial membrane (curves 2–4). Curve 1 was obtained in the absence of penetrating anions. It can be seen that 10 mM

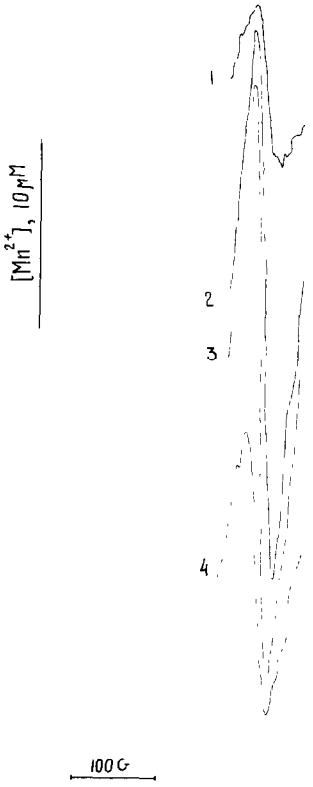

Fig.4. The third line of the ESR spectra of Mn^{2+} inside the submitochondrial particles. The medium contained 0.25 M sucrose, 10 mM HEPES–H_2SO_4, pH 7.0, 3 mM EDTA, 10 mM $MgSO_4$, 5 mM ATP, Mn-SMP 10 mg/ml and additions of different salts. Curve 1, none; curve 2, 25 mM KCl; curve 3, 10 mM KNO_3; curve 4, 1 mM KCNS.

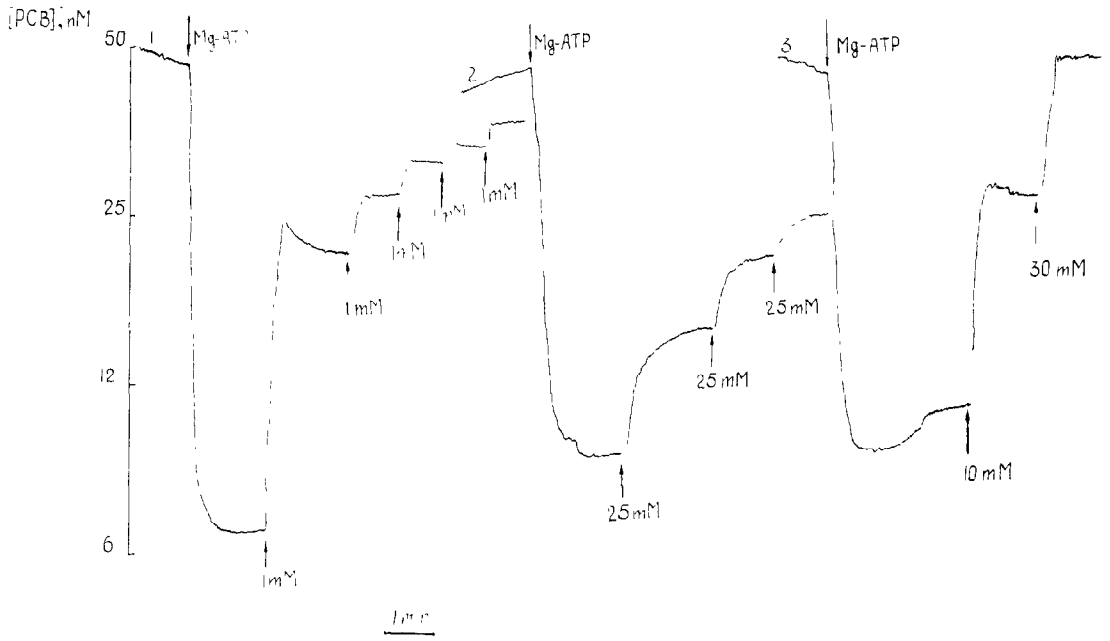

Fig.5A

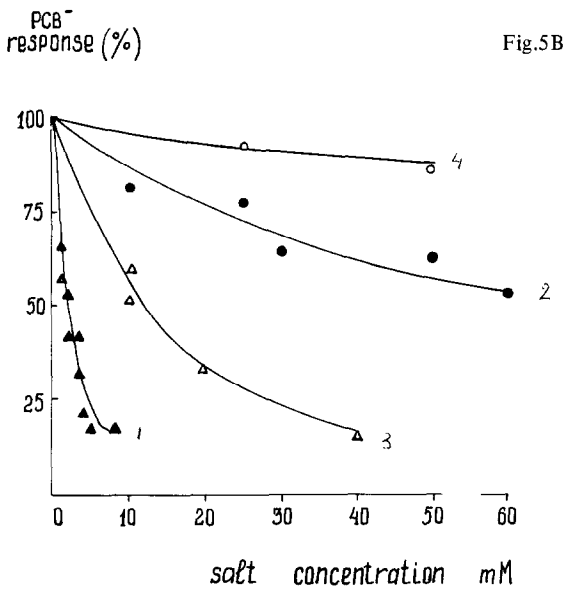

Fig.5B

Fig.5. The inhibiting effect of various anions on ATP-hydrolysis-coupled generation of the membrane potential. For conditions see fig.4, but protein was 1 mg/ml. (A) Arrows show additions of the salts. Curve 1, KCNS; curve 2, KCl; curve 3, KNO_3. (B) Curve 1, KCNS; curve 2, KCl, curve 3, KNO_3; curve 4, K_2SO_4.

KNO_3 is as effective in inducing an ATP-dependent increase in the free Mn^{2+} concentration inside the particles as is 25 mM KCl (fig.4, curves 2 and 3). This result does not seem surprising, since those concentrations of the salts cause roughly comparable decreases of the ATP-dependent membrane potential in the submitochondrial particles. (fig.5A,B). This decrease in the membrane potential in the presence of the penetrating anions is due to the conversion of the membrane potential into ΔpH on the mitochondrial membrane [12]. ΔpH formation which appears as a drop in pH inside the particles induces an observed increase in the free Mn^{2+} concentration (fig.4).

It appeared, quite surprisingly, that CNS^-, which are well known as penetrating anions, effectively abolished the membrane potential (fig.5) but did not induce a great increase in the free Mn^{2+} concentration inside the particles (fig.4, curve 4). The latter observation may be a result of a complex formation between Mn^{2+} and CNS^- inside the particles.

In the experiments shown in fig.3–5, salts were added to the incubation mixture immediately before measurements were made. The same results were obtained if these salts were present in a medium used for ultrasonic treatment of mitochondria and for sub-

sequent washing of Mn-SMP. In all cases, sulfate, which does not transform the membrane potential into ΔpH, did not induce an ATP-dependent increase in the free Mn^{2+} concentration inside the particles either.

The data obtained indicate that the increase in the free Mn^{2+} concentration inside the particles in the presence of penetrating anions (Cl^-, NO_3^-, CNS^-) and ATP, is the result of energy-dependent acidification of the submitochondrial particles' internal space. Using the ESR technique to measure this increase in the free Mn^{2+} concentration, it is possible to follow ΔpH generation on the particles' membrane.

Acknowledgement

The authors would like to express their thanks to Professor V. P. Skulachev for discussion of the results.

References

[1] Gunther, T. E. and Puskin, J. S. (1972) Biophys. J. 12, 625–635.
[2] Puskin, J. S. and Gunther, T. E. (1973) Biochem. Biophys. Res. Commun. 51, 797–803.
[3] Gunther, T. E., Puskin, J. S. and Russell, P. R. (1975) Biophys. J. 15, 319–332.
[4] Bragadin, M., Dell'Antone, P., Pozzan, T., Volpato, O. and Azzone, G. F. (1975) FEBS Lett. 60, 354–358.
[5] Pozzan, T., Bragadin, M. and Azzone, G. F. (1976) Eur. J. Biochem. 71, 93–99.
[6] Dell'Antone, P., Volpato, O., Ronconi, G. and Pregnolato, L. (1977) FEBS Lett. 81, 243–248.
[7] Crane, F. L., Glenn, J. L. and Green, D. E. (1956) Biochim. Biophys. Acta 22, 475–480.
[8] Hansen, M. and Smith, A. L. (1964) Biochim. Biophys. Acta 81, 214–222.
[9] Grinius, L. L., Jasaitis, A. A., Kadziauskas, Yu. P., Liberman, E. A., Skulachev, V. P., Topali, V. P., Tsofina, L. M. and Vladimirova, M. A. (1970) Biochim. Biophys. Acta 283, 442–455.
[10] Reed, P. and Lardy, H. A. (1972) J. Biol. Chem. 247, 6970–6977.
[11] Weiner, M. W. (1975) Am. J. Physiol. 228, 122–126.
[12] Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 7, 471–484.