Interaction of Mn^{++} with Submitochondrial Particles

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Received February 26, 1985

Abstract

The aerobic uptake of Mn^{++} in rat liver mitochondria gives rise to an internal ESR signal which is the sum of two components: a sextet signal corresponding to hydrated Mn^{++} and a single line representing the spin exchange signal. While no problems arise from the interpretation of the sextet signal, the spin exchange component has been attributed either to Mn^{++} binding to specific energy-dependent membrane sites or to $Mn_3(PO_4)_2$ precipitate. The aim of the present paper is to study the interaction between Mn^{++} and the 'internal' mitochondrial membrane surface without interference due to phosphate presence (and to external Mn–EDTA complex signal), accepting that submitochondrial particles are 'inside-out' membranes in which the internal mitochondrial membrane surface is in contact with the external medium.

Introduction

The energy-conservation mechanism in rat liver mitochondria involves H^+ extrusion and H^+ electrochemical gradient $\Delta\psi$ [1]. Since internal membrane is permeable to divalent cations, [2, 3], (Ca^{++}, Sr^{++} and Mn^{++}), the electrical component of this gradient, $\Delta\psi$, gives rise to accumulation of divalent cations in the matrix. Mn^{++} is a paramagnetic ion and its ESR spectrum allows it to be distinguished among aqueous hydrated ion, membrane binding, complex formation and Mn-phosphate precipitate [4–8]. In the case of Mn^{++} accumulation in the mitochondrial matrix, the spectrum appears as the sum of a sextet due to hydrated Mn^{++} and a single line component denoted as E. This component has been previously interpreted either as due to cation binding to energy-dependent sites [4–6] or to $Mn_3(PO_4)_2$ precipitate [7, 8]. The major argument supporting the first interpretation was that the width of the E component changes with the accumulation extent, reflecting the presence of a large number of sites capable of complexing divalent cations in the region of high local concentration. The interpretation in favour of the $Mn_3(PO_4)_2$ precipitate is supported by the following evidence:

(1) The component does not appear in the presence of inhibitors of P_i transport.

(2) The width of the Spin Exchange signal of $Mn_3(PO_4)_2$ in solution is not constant but depends on the aggregation state of the Mn–P_i complex and therefore on the kinetics of the aggregation. The higher the Mn:phosphate ratio, the faster the kinetics.

(3) Since the width of the line is constant (about 220 gauss), this allows a correct measurement of the amount of Mn^{++} in the E component. This value corresponds well to the amount of endogenous P_i (about 60 n mol/mg protein).

Following the first interpretation, the E component is due to the binding of Mn^{++} to energy-dependent sites of the inner mitochondrial membrane. This interpretation, however, does not explain whether the sites are new sites appearing under energization or whether they pre-exist before energization and consequent internal ion accumulation. In this case the binding may be due to high Mn^{++} concentration in the internal aqueous matrix.

In this paper the aim is to provide new evidence in support of the Mn-phosphate interpretation, verifying that even if interaction between accumulated Mn^{++} and internal sites may exist, the sites are not solely due to energization and that the binding of Mn^{++} is of such an extent, that it does not influence the $Mn_3(PO_4)_2$ ESR signal. For a simple study of this interaction, submitochondrial particles have been utilized. In this system, as largely demonstrated [9, 10], all biological functions are inverted and the internal surface of the mitochondrial membrane is in contact with the external medium. This fact permits the study of the interaction of Mn^{++} with the 'internal' surface of the mitochondrial membrane simply without any interference due to phosphate presence.

Experimental

Submitochondrial particles have been prepared by sonication of rat liver mitochondria following the

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0020-1693/85/$3.30

Racker's procedure [11]. To leave out EDTA, the last two washed were performed on EDTA-free medium.

In order to reproduce the conditions of the internal aqueous matrix in energized mitochondria as closely as possible, all experiments were performed on a medium at pH 8.

All chemicals used were of analytical grade.

ESR spectra at X band (9100 Mc/s) were recorded with a Varian V-4502 spectrometer. The microwave frequency was approximately 9.5 G Hz. The modulation amplitude, recorder time constant and scanning rate, were as follows: 16 gauss, 0.3 s, 250 gauss/min. Since the ESR technique does not allow the detection of slight variations in the concentrations of aqueous Mn^{++}, all experiments (and those of Fig. 3 exclusively) were followed, not only by means of the ESR technique, but spectrophotometrically as well using Antipyrilazo III as Mn^{++} sensitive probe [12] by means of an Aminco Dual Wavelength spectrophotometer (540–600 the used wavelengths) equipped with magnetic stirring. The use of Antipyrilazo III permits operating with 1 mg ml^{-1} of submitochondrial particles (instead of 12 mg ml^{-1} as in the case of Figs. 1 or 2). This procedure avoids spurious interpretations due to the high protein concentration which causes a fast oxygen consumption and consequent de-energization.

The simulations of Figs. 1 and 2 were carried out with a PDP 11/23 Digital Computer, assuming the large Mn^{++} component is due to a single derivative Lorentzian curve with a line width of 500 gauss.

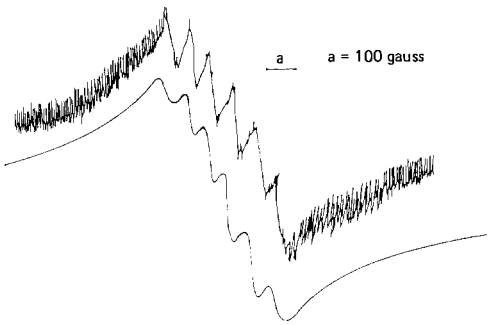

Fig. 1. The figure shows the ESR spectrum of Mn^{++} due to interaction with submitochondrial particles (upper curve) and the simulated spectrum (lower curve). The medium contained: 0.22 M Sucrose, 40 mM Choline, 10 mM Tris-Cl pH 8, 0.1 mM Antipyrilazo III, 0.25 μM Ruthenium Red, 5 mM succinate. Submitochondrial particles 12 mg/ml and Mn^{++} 1 mM. The same spectrum was obtained after de-energization with FCCP or Dinitrophenol as uncouplers. The same experiment was confirmed spectrophotometrically using Antipyrilazo III as Mn^{++} sensitive probe even at different Mn^{++}:protein ratios (see 'Experimental').

Results and Discussion

Figure 1 shows an ESR spectrum due to interaction of Mn^{++} with submitochondrial particles. Since the spectrum is not modified if the submitochondrial particles have been energized, (even at different Mn:protein ratios), the presence of energy-dependent sites may be excluded. Since the energization may involve little changes in concentration of Mn^{++} in solution, which cannot be detected by ESR, and to avoid anaerobiosis due to the high protein concentration in the ESR experiments, the experiment of Fig. 1 has been confirmed spectrophotometrically (see 'Experimental') at any Mn:protein ratio. Even in this case, the Mn^{++} free concentration does not change on energization.

The type and the shape of the ESR spectrum shows however, that an interaction between Mn^{++} and submitochondrial particles occurs. The spectrum due to interaction of Mn^{++} and submitochondrial particles may be ascribed to the sum of two components:

(1) a six line component due to aqueous Mn^{++}.

(2) a large single line (about 500 gauss) centered at g = 2 that may be due to Spin Exchange interaction which can be well simulated using a Lorentzian derivative.

To clarify the nature of the nonaqueous component, the experiment in Fig. 2 shows the effect of

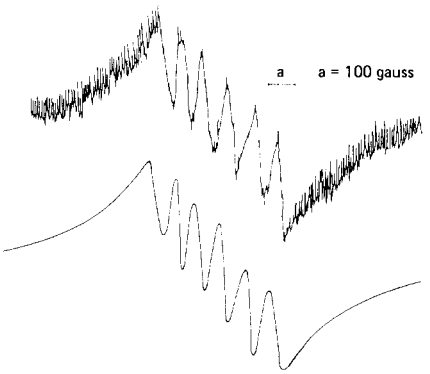

Fig. 2. The figure shows the competitive effect of Mg^{++} (2 mM) in the same conditions as in Fig. 1. The upper curve refers to the experimental and the lower to the simulated spectrum.

a competitive ion as Mg^{++}. Under the same conditions as in Fig. 1 the addition of Mg^{++} clearly shows an enhancement of the aqueous component (the same effect may be obtained without Mg^{++} using higher Mn:protein ratios than in Fig. 1).

If the single line large component were due to Spin Exchange interaction, the competitive effect of Mg^{++}, would give rise to a line width increase (more than 500 gauss) [5]. This effect does not

occur, as is evident from the good simulation obtained using a 500 gauss Lorentzian derivative for both experiments of Figs. 1 and 2. Since in all cases, at all Mn:protein ratios, a good simulation with more or less than 500 gauss has never been obtained, we can exclude the existence of strong interacting sites giving rise to spin exchange interaction. The large single line component, even if well reproduced by a Lorentzian, may thus be due to a Mn complex formation with binding sites.

In order to establish the extent of the interaction between Mn^{++} and 'internal' mitochondrial membrane, a Scatchard plot as in Fig. 3 has been performed. From the plot the presence of a straight line suggests the presence of only one type of site interacting with Mn^{++}. This fact may thus suggest that the nonaqueous component may be due to a complex formation of Mn^{++} with the membrane arising from only one type of binding site. From the x axis intercept it is possible to calculate that the amount of sites is about 5–6 n mol/mg protein. Even taking into account the hypothesis that during active uptake of Mn^{++} in rat liver mitochondria, all sites were saturated, the value is largely negligible with respect to the 60–70 n mol under precipitate form [8]. Considering that the 500 gauss component is much broader than those due to Mn–phosphate, we can conclude that the interaction of Mn^{++} with the 'internal' mitochondrial membrane, gives rise to an ESR signal which is largely negligible in comparison with the signal due to $Mn_3(PO_4)_2$.

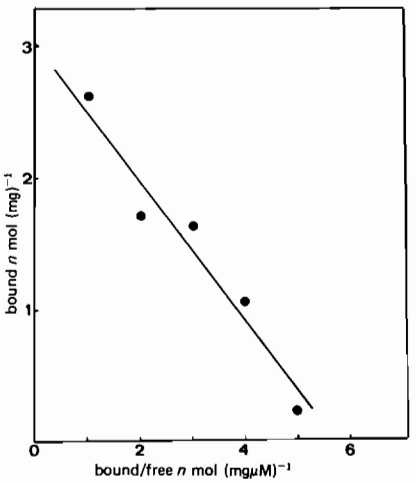

Fig. 3. Scatchard plot to estimate the binding sites of Mn^{++} with submitochondrial particles. Medium as in Fig. 1. Protein concentration 1 mg/ml and Antipyrilazo III 0.1 mM.

References

1 D. G. Nicholls, 'Bioenergetics', Academic Press, London and New York, 1980.
2 F. L. Bygrave, *Biol. Rev., 53,* 47 (1978).
3 N. E. Saris and K. E. O. Akermann, *Curr. Top. Bioenerg., 10,* 103 (1980).
4 T. E. Gunther and J. S. Puskin, *Biophys. J., 12,* 625 (1972).
5 T. E. Gunther, J. S. Puskin and P. R. Russel, *Biophys. J., 15,* 319 (1975).
6 J. S. Puskin, T. E. Gunther and M. T. Coene, *Eur. J. Biochem., 106,* 425 (1980).
7 T. Pozzan, M. Bragadin and G. F. Azzone, *Eur. J. Biochem., 71,* 93 (1976).
8 M. Bragadin, T. Pozzan and G. F. Azzone, *Eur. J. Biochem., 134,* 385 (1982).
9 C. P. Lee and L. Ernster, in J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater (eds.) 'Regulation of Metabolic Process in Mitochondria, Vol. 7', BBA Library, Elsevier, Amsterdam, 1966, p. 218.
10 E. Racker, *J. Gen. Physiol., 54,* 385 (1969).
11 E. Racker, *Proc. N. Y. Acad. Sci., 48,* 1659 (1962).
12 A. Scarpa, F. J. Brinley and A. Dubjack, *Biochemistry, 17,* 1378 (1978).