ESR DETERMINATION OF Mn^{++} UPTAKE AND BINDING IN MITOCHONDRIA

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1. Introduction

Active transport in energy transducing membranes involves the utilization of metabolic energy to move ions against electrochemical gradients. In the case of the divalent cations which are actively transported by mitochondria, namely Ca^{++}, Mn^{++} and Sr^{++} [1], the problem arises as to the determination of the matrix concentration of osmotically active cations. Massari et al. [2] have calculated the ion uptake from absorbancy changes. Gunther and Puskin [3–5] have used the ESR signal of Mn^{++} to distinguish between free and bound cations and to calculate the matrix concentration of free Mn^{++}. According to Gunther and Puskin [3], during active uptake the Mn^{++} spectrum can be split into two components E and S belonging to membrane bound and matrix free Mn^{++}, respectively. The binding of divalent cations to the membrane has also been followed qualitatively through the enhancement of the fluorescent chelate of tetracycline with divalent cations [6]. In the present paper the changes of e.s.r. signal of Mn^{++} during active uptake have been correlated with absorbance and matrix volume changes. On the basis of the combined volume and e.s.r. data a procedure is described for the determination of free Mn^{++} concentration in the mitochondrial matrix.

2. Experimental

Rat liver mitochondria have been prepared according to standard procedures. The last two washes were performed in an EDTA free medium. The protein determination was made with the biuret procedure. Photometric measurements were performed with an Eppendorf photometer equipped with a stirring device at 546 nm. All chemicals used were analytical grade. E.s.r. spectra at X band (9100 Mc/sec) were registered with a Varian V-4502 spectrometer. All measurements were made at room temperature. To avoid anaerobiosis and to obtain good reproducibility of the spectra we have used a continuous flow apparatus [7] and a quartz capillary tube permanently positioned into the resonance cavity. The microwave frequency was approx. 9.5 GHz. The modulation amplitude (M), recorder time constant (tc), and scanning rate (SR) were as follows: M 16 gauss; tc 0.3 sec; SR 250 gauss/min. The height of the derivative curve from the maximum to the minimum was found to be a linear function of the concentration of manganous ion in the range investigated, namely 5–500 μM, and it was therefore unnecessary to integrate the curves to obtain the concentration. The matrix volume was determined both gravimetrically and with ^3H_2O. The external space in the mitochondrial pellet was determined with ^{14}C dextrane, mol. wt 60 000 [8].

3. Results

The interaction of Mn^{++} with P-lipids [9] and proteins [10] results in a line broadening, with decrease of the intensity of the e.s.r. signal. Fig.1 shows that addition of mitochondria to a solution containing free Mn^{++} in water resulted in a decrease of the e.s.r. signal. The decrease was proportional to the amount of mitochondria (not shown). Fig.1 shows also that the subsequent addition of Mg^{++} resulted in a complete restoration of the e.s.r. signal.

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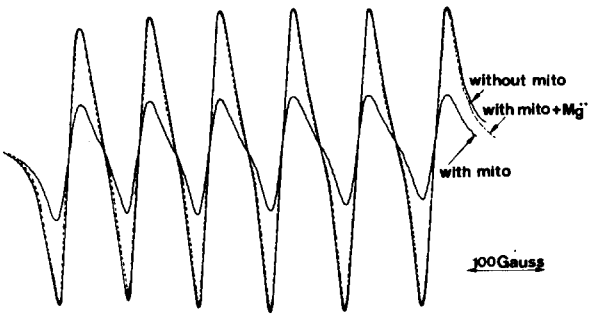

Fig.1. Passive interaction of Mn^{++} with mitochondria. Release of Mn^{++} induced by Mg^{++}. Medium: 0.25 M sucrose, 10 mM Hepes, pH 6.5, 3 μM FCCP, 3 μM rotenone, 25 μM $MnCl_2$. When indicated, 5 mg/ml mitochondrial protein and 10 mM $MgCl_2$.

The restoring effect of Mg^{++} tends to be less complete parallel to the increase of the mitochondrial protein. Extrapolation to infinite protein concentration results in almost complete abolition of the e.s.r. signal. The number of Mg^{++}-sensitive binding sites for Mn^{++} amount to 20–30 nmol X mg protein^{-1}, in accord with measurements carried out with this [11] and other techniques [12]. Because of the Mg^{++} sensitivity and independence from energy supply, the decrease of e.s.r. signal can be attributed to passive binding of Mn^{++} at the outer mitochondrial surface, presumably with P-lipids [13].

Fig.2A shows that addition of succinate to rotenone treated mitochondria, incubated in the presence of Mn^{++} caused a marked decrease of the height of the e.s.r. signal. The succinate effect was completely reversed by FCCP. Similar observations have been reported by Chappell et al. [11] and by Gunther and Puskin [3]. Both groups interpreted the effect as due to binding of Mn^{++} to the membrane. Gunther and Puskin were also able to show a spin exchange for the membrane bound Mn^{++}. This effect is not apparent in fig.2. This is due to our using a much lower sensitivity of the spectrometer. The lower sensitivity was selected in order to follow the free Mn^{++} without the overlapping signal of bound Mn^{++}. Fig.2A shows also that addition of EDTA, after succinate, caused a complete quenching of the e.s.r. signal. The quenching after EDTA is due to chelation of Mn^{++} free in the incubation medium. Thus the quenching of the Mn^{++} e.s.r. signal is due, in part, to active uptake and binding of Mn^{++} to the membrane, and, in part, to chelation by EDTA of the Mn^{++} in the outer aqueous phase. From fig.2A it appears that, in the absence of acetate the concentration of free Mn^{++} in the matrix is negligible. Note that under the selected experimental conditions, it was impossible to determine with accuracy an uptake of free Mn^{++} lower than 1 nmol X mg protein^{-1}. The amount of actively bound Mn^{++}, under the conditions of fig.2A was about 80–100 nmol X mg protein^{-1}, in accordance with previous reports [3,14].

Fig.2B shows that, also in the presence of 10 mM acetate, succinate induced a decrease of the Mn^{++} e.s.r. signal. However the subsequent addition of EDTA did not result in a complete abolition of the Mn^{++} e.s.r. signal as in fig.2A without acetate. 10 mM acetate was

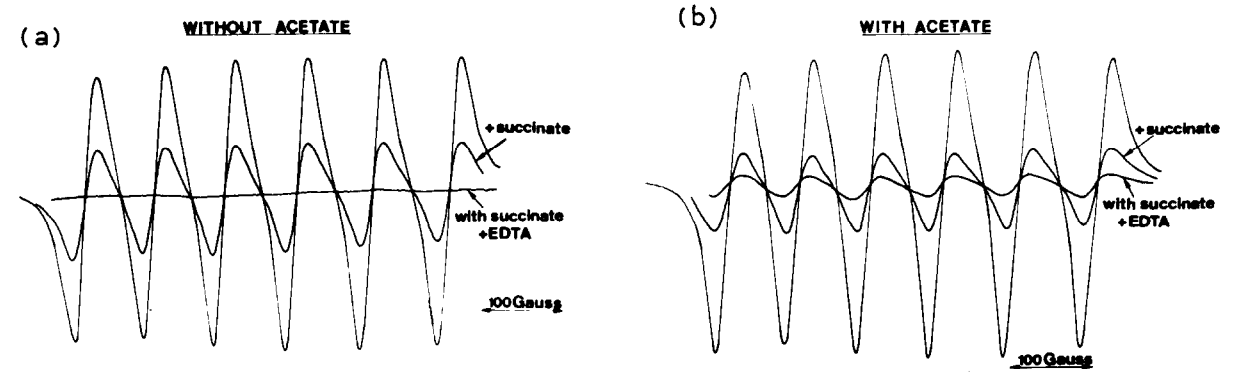

Fig.2.(a,b) Effect of EDTA on the Mn^{++} signal in the absence and presence of acetate. Medium: 0.25 M sucrose, 10 mM Hepes, pH 6.5, 3 μM rotenone, 600 μM $MnCl_2$. When indicated, 5 mg/ml mitochondrial protein, 4 mM succinate-Tris, 10 mM acetate and 1 mM EDTA.

selected because it was found the concentration inducing the maximal increase of matrix volume in the range 100–200 nmol Mn^{++} X mg protein^{-1} (Azzone et al., in preparation) [15]. The line width of the Mn^{++} signal after EDTA was about 30–35 gauss, as compared to about 25 gauss of line width for Mn^{++} free in water. However a number of factor may affect the line width of free Mn^{++} [16].

Gunther and Puskin assumed that the residual Mn^{++} e.s.r. signal in the presence of EDTA is to be attributed to free Mn^{++} in the mitochondrial matrix and calculated [5] from the height of Mn^{++} e.s.r. signal in the presence of EDTA, the concentration of free Mn^{++} in the matrix. A number of questions here arise, namely the assignment of the e.s.r. signal in the presence of EDTA to matrix free Mn^{++}, the effect of EDTA on the matrix concentration of free Mn^{++}, and the osmotic equilibration of the matrix during Mn^{++} uptake.

Rasmussen et al. [17] showed that uptake of Ca^{++} in the presence of acetate resulted in a large decrease of absorbance of the mitochondrial suspension. This was interpreted as mitochondrial swelling due to the penetration of osmotically active material in the mitochondrial matrix. Fig.3 shows the changes of matrix volume and absorbance of the mitochondrial suspension as occurring during uptake of Mn (acetate)$_2$ in the presence of variable amounts of Mn^{++}. It is seen that the mitochondrial volume increased from 0.4 μl X mg protein without cation uptake to 1.5 μl X mg protein^{-1} at maximal cation uptake, fig.3. The volume increase was negligible below 50 nmol X mg protein^{-1} and reached a maximum at about 200 nmol X mg protein^{-1}. There was a very good correlation

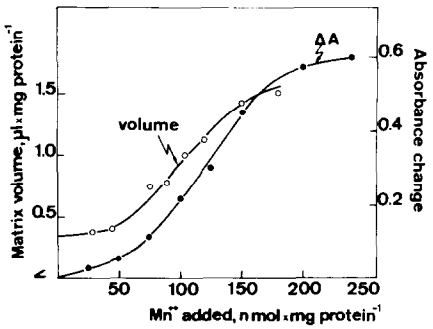

Fig.3. Matrix volume changes and absorbance changes during Manganese acetate accumulation. Medium as in fig.2, except Mn^{++} as indicated.

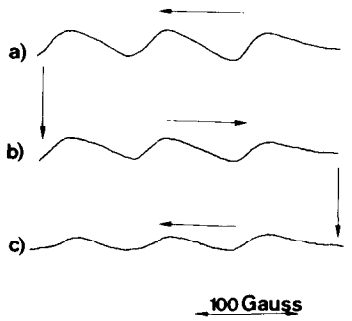

Fig.4. Typical kinetics of EDTA – induced decay of e.s.r. signal. Scan time 250 gauss/min. Scanning of the spectrum started 1 min after addition of EDTA.

between absorbance and volume determinations [2].

Fig.4 shows that addition of EDTA resulted in a decay of the e.s.r. signal of free Mn^{++} in the matrix. This is due to an EDTA induced efflux of cation from the matrix. An EDTA induced efflux of membrane bound Ca^{++} has been observed previously [6,18]. Fig.5 shows a correlation between e.s.r. and absorbance data during EDTA and FCCP + EDTA induced Mn^{++} efflux. The FCCP induced efflux was much faster. However in both cases there was a good correlation between absorbance and e.s.r. data. The data of figs.3 and 5 support the validity of the absorbance measurement to follow the kinetics of the penetration

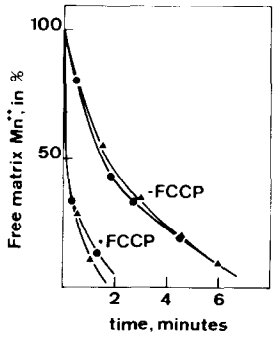

Fig.5. Correlation between e.s.r. and photometric data concerning the amount of free Mn^{++} in matrix. Medium: 0.25 M sucrose, 10 mM Hepes, 3 μM rotenone, 300 μM $MnCl_2$, 10 mM Na-acetate, 4 mM succinate, 2.8 mg/ml mitochondrial protein. 0.5 μM FCCP + 1 mM EDTA, or 1 mM EDTA were added after completion of uptake. (▲) % Absorbance increase. (●) % E.s.r. signal amplitude.

Table 1
Uptake and concentration of Mn^{++} in matrix

Mn^{++} added (nmol × mg protein)	Mn^{++} uptake (free)	Matrix volume (μl × mg protein^{-1})	Concentration (mM)
40	∿ 0.8	0.4	2.0
60	3.0	0.5	6.0
120	10.6	1.14	9.3
180	13.2	1.57	8.4

of osmotically active material into the matrix. Furthermore they support strongly the conclusion of Puskin and Gunther [4] that the S component of the e.s.r. Mn^{++} signal in the presence of EDTA is due to free cation in the matrix.

Table 1 shows the concentration of free Mn^{++} in the matrix, as calculated from the e.s.r. data corrected for the increase of matrix volume. The concentration of free Mn^{++} in the matrix was very low at low cation/protein ratios and then increased above 40–50 nmol × mg protein^{-1}. However even at high cation/protein ratios the matrix concentration of Mn^{++} was never higher than 10 mM. These values are 4–5 times lower than those of Puskin and Gunther [5] due to the parallel increase of matrix volume from 0.4 to 1.57 μl × mg protein^{-1}.

Discussion

Thermodynamic and molecular mechanisms of active transport depend on the knowledge of the osmotic work performed during transport and thus on the determination of the electrochemical ion gradients. The interaction with the membrane sites is weak in the case of the univalent and strong in the case of the divalent cations. This renders questionable any assumption on the osmotic activity of divalent cations in the absence of direct measurement. Gunther and Puskin introduced the e.s.r. technique to measure the concentration of free Mn^{++} in the matrix by taking advantage of the e.s.r. signal of the hexahydrate Mn complex in water after quenching with EDTA the signal of the residual Mn^{++} in the outer space. The correlation found in the present work between height of e.s.r. signal in the presence of EDTA and extent of swelling, measured either by absorbance changes and by direct determination of the matrix volume, is in accord with the conclusion that the S-component of the e.s.r. signal of Mn^{++} can be used to measure the amount of free Mn^{++} in the matrix. While the approach of Gunther and Puskin [5] is valid in principle two corrections need to be introduced to calculate the matrix concentration of Mn^{++}. First, since EDTA caused an efflux of Mn^{++} the height of the signal after EDTA must be extrapolated to zero time. Second, the increase in matrix volume due to the penetration of Mn (acetate)$_2$ has to be taken into account. Gunther and Puskin [5] calculated that the amount of water penetrating into the matrix is negligible, since they assumed that during MnAc accumulation one Ac^- enters with each Mn $(H_2O)_6{}^{++}$. This calculation takes into account the amount of water complexing with Mn^{++} but ignores the amount of water moving because of osmotic equilibration through a membrane highly permeable to water. It may be easily calculated that while the H_2O moving as a complex together with 100 nmol Mn^{++} × mg protein^{-1} is 600×10^{-9} mol, that moving osmotically is hundred times higher namely 600×10^{-7} mol, in a medium 270 mosm. The correction for the increase of matrix volume is the main reason for the discrepancy between Puskin and Gunther [5] and present data. Puskin and Gunther also report an increase of the amount of matrix free Mn^{++} following an increase of the acetate concentration in the range 10–100 mM. Our determinations [15] show that, above 10 mM acetate, there is no further increase of the matrix volume. Moreover, in the present work, at variance from Puskin and Gunther [5] it was impossible to detect a signal of free Mn^{++} in the matrix in the absence of acetate. This may be due to our using a lower succinate concentration or to a lower matrix concentration of metabolite anions. We have also found that complete abolition of the signal of free Mn^{++} requires an EDTA concentration higher than stoicheometric in the presence of mitochondria.

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