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EVIDENCE FOR THE TRANSPORT OF MANGANOUS ION AGAINST AN ACTIVITY GRADIENT BY MITOCHONDRIA

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SUMMARY

Mn^{2+} taken up by mitochondria through the often discussed energy dependent process has been previously shown to be separable by EPR spectroscopy into two spectral fractions (T. E. Gunter and J. S. Puskin, *Biophys. J.*, 12 (1972) 625). One of these spectral fractions shows the characteristics of spin exchange. The other fraction, comprising roughly 10 % of the spectrally observed Mn^{2+}, shows a hyperfine sextet.

Evidence is presented supporting the view that under conditions of maximum uptake of Mn^{2+} by the mitochondria in the absence of exogenous phosphate, the bulk of the hyperfine sextet fraction is in the $Mn^{2+}(H_2O)_6$ form.

Spectral data is then used to show that if this view is correct, this fraction is found within mitochondria under conditions where it can be shown to have been accumulated against an activity gradient.

Spectral line width data for this fraction is interpreted in such a way as to provide an upper limit to intramitochondrial local viscosity where this hexahydrate form of manganese is present. This upper limit is approximately 1.5 cP at 37 °C and 0.25 M external osmolarity for example.

INTRODUCTION

It was reported that the EPR spectrum (at x-band) of Mn^{2+} transported by the energy-dependent process into mitochondria consists of two components[1]:

(a) A single line, E, near $g = 2$, exhibiting spin exchange characteristics. The peak-to-peak line width is, as a rule, narrower than the hyperfine envelope and diminishes as uptake increases.

(b) A hyperfine sextet, S, with splittings very close to those of Mn^{2+} in distilled water, but encompassing a range of line widths from 30 to 52 Oe or more depending on experimental conditions.

Previous work was concerned primarily with the properties of E, which makes up the bulk of the intensity. Here we present evidence that under certain conditions a significant fraction of S arises from the free $Mn^{2+}(H_2O)_6$ complex, henceforth referred to as "free Mn^{2+}" and draw conclusions on the basis of that inference.

The EPR spectrum of free Mn^{2+} in distilled water at 23 °C consists of six

hyperfine lines with an average splitting of $\simeq$ 95 Oe and with widths ranging from 23 Oe for the fourth line to 26 Oe for the first line. Several studies of dilute Mn^{2+} and related transition ions in aqueous solution, with and without additional solutes, and in a number of solvent mixtures, have recently been published[2-6]. A brief review of some of the conclusions of these investigators will facilitate the interpretation of the data presented here.

Complexes of Mn^{2+} in solution with relaxation times long enough to allow the resolution of hyperfine splitting generally have high (octahedral or tetrahedral) symmetry. The line widthsof the sextet are primarily determined by fast spin relaxation resulting in energy level broadening as required by the uncertainty principle. Mn^{2+} is preferentially solvated by six water molecules even when only trace amounts of water are present in mixed solvents[2].

It is well established that the mechanism of relaxation, at temperatures relevant for biological studies, is modulation of the zero field splitting arising from transient distortions of the outer hydration sphere by collisions with solvent molecules. Whether the modulation mechanism is Browian rotation or collision induced fluctuations in crystal field, theory predicts and experiment confirms that the line width increases with viscosity, gradually leveling off to a constant value characteristic of the glassy state[2,3,5,6]. The functional dependence of line width on temperature is complex[2,3,7], but in the range of biological interest the predominant effect of temperature variation on line width is through change in viscosity. It should be noted that the viscosity referred to here is local viscosity as would be determined, *e.g.* from a measurement of the rate of fluorescence depolarization in the medium and not the macroscopic viscosity as would be measured by say an Ostwald viscometer. The relaxation rate of Mn^{2+} in aqueous solution can also be enchanced by transient disruptions of their hydration spheres by formation of short lived complexes with anions. The increments in line width originating in this way from the concentrations of small monovalent and divalent cations present in the mitochondria is estimated to be less than 0.5 Oe and 2.0 Oe, respectively (refs 2,7; and T. E. Gunter and J. S. Puskin, unpublished results). Negative groups on macromolecules may also contribute broadening through this mechanism; consequently, estimates of local viscosity derived from Mn^{2+} line width data must be regarded as upper limits unless the effect of anions on line width can be shown to be negligible or unless the data can be corrected for these effects.

Qualitatively, a spectrum such as S is what one would expect to arise from a free Mn^{2+} fraction influenced by viscosity broadening of the type discussed above. This is particularly true of the narrowest observed lines of S ($\simeq$ 30 Oe) which, as we shall see, correspond to quite reasonable intramitochondrial viscosities. No bound Mn^{2+} complexes are expected to occur inside mitochondria that would give rise to a sextet with lines as narrow as this. When Mn^{2+} is bound to proteins[8] or phospholipids[9], the spectra do not usually show hyperfine structure. In a few exceptional cases, *e.g.* jack bean protein, there is an observable sextet but the peak-to-peak line widths are $\simeq$ 40 Oe. Mn^{2+}--adenine nucleotide and citrate chelates exhibit broad ($\geqslant$ 40 Oe) hyperfine sextets.

METHODS

The methods for investigating Mn^{2+} transport under limited loading conditions

were similar to those described in ref. 1 except that the mitochondrial preparation was stabilized by 1 mg/ml bovine serum albumin and each aliquot included 0.1 mM ATP to stimulate uptake. As in ref. 1, the pH of the mitochondrial suspension medium was around 7.3. Again as in ref. 1, EDTA was employed after 10 min of exposure to Mn^{2+} in order to effectively remove the spectral component contributed by external Mn^{2+} by greatly broadening the lines. Acetate was sometimes substituted for chloride as an anion. Crystallized bovine serum albumin was obtained from Pentax. In certain experiments concentrated bovine serum albumin solutions were used to simulate the viscosity of concentrated soluble protein solutions thought to be present in the mitochondrial matrix. For these bovine serum albumin solutions the pH was adjusted to below 5.0 in order to eliminate Mn^{2+} binding.

Viscosities were determined by comparing the linewidth of the Mn^{2+} hexahydrate in the mitochondrial sample with that in a sucrose solution where viscosity was known.

RESULTS AND DISCUSSION

As delineated in the Introduction, narrow lines of S (30–35 Oe) are likely to correspond to a free Mn^{2+} fraction inside the mitochondria. Such spectral components occurred whenever uptake, under limited loading conditions, was large (> 70 nmoles Mn^{2+}/mg mitochondrial protein) as reflected by strong spin exchange (line widths of $E < 260$ Oe). The fact that the lines of the sextet sometimes appear broader under conditions of smaller uptake is consistent with this hypothesis. In general, S may consist of the super position of a component due to free Mn^{2+} with components due to bound forms with resolvable but broader hyperfine lines. The measured peak-to-peak line widths of the sextet will be intermediate between those of the two components. As uptake increases the ratio of free to bound Mn^{2+} increases, and because the narrowest component tends to dominate the EPR spectrum, the observed line widths approach those of the free form, *viz.* $\simeq$ 30 Oe.

If it is correct that S, under conditions of large uptake, arises primarily from a free Mn^{2+} fraction in a viscous medium, it should be possible to change the line width by modifying the viscosity, *i.e.* as the temperature is raised the line width should decrease. Since mitochondria act as osmometers, a reduction of external osmolarity should dilute the intramitochondrial protein solution, resulting in a less viscous medium and less line broadening. Conversely, the degree of broadening, under fixed external conditions, can be used to provide an estimate of the local internal viscosity or, as discussed above, an upper limit to this viscosity. Such line width dependences have been observed and some typical results are summarized in Table I.

To test whether these viscosity variations are reasonable, the intramitochondrial medium, which is believed to be a highly viscous protein solution, was simulated by a concentrated solution of bovine serum albumin containing 1 % KCl, 1 mM $MnCl_2$. The concentration of bovine serum albumin was adjusted so that Mn^{2+} line widths of $\simeq$ 30.5 Oe were observed at room temperature; this bovine serum albumin concentration, corrected for trapped solvent in the powder, was approximately 30 %. The line width as a function of temperature and dilution was then recorded. The temperature dependence, along with comparable data for a water–glycerol mixture and for the S component of Mn^{2+} in mitochondria is shown in Fig. 1. The dependence

TABLE I

INTRAMITOCHONDRIAL VISCOSITIES AS INTERPRETED FROM LINE WIDTH DATA FOR MANGANESE HEXAHYDRATE IN THE INTRAMITOCHONDRIAL MEDIUM UNDER A VARIETY OF CONDITIONS OF TEMPERATURE AND OSMOLARITY OF THE MEDIUM EXTERNAL TO THE MITOCHONDRIA (SUCROSE)

No error limits were given for these viscosities since these numbers must be interpreted in the strict sense as upper limits for viscosity. Under conditions where anion effects are negligible, it should be possible to determine viscosities within about 15 % using this technique.

Temperature (°C)	*External osmolarity* (*molar*)	*Line width* (*Oe*)	*Viscosity* (*cP*)
8	0.16	33.2 ± 0.5	3.8
23	0.16	28.0 ± 0.5	1.9
37	0.16	25.1 ± 0.5	1.4
8	0.33	34.3 ± 0.5	4.4
23	0.33	30.9 ± 0.5	2.7
37	0.33	26.5 ± 0.5	1.6

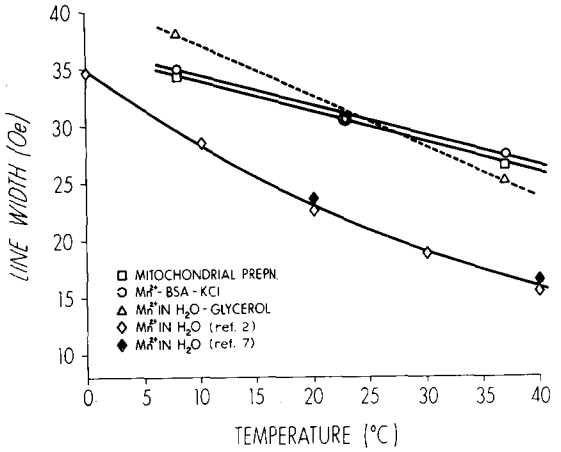

Fig. 1. Peak-to-peak line width *vs* sample temperature for a series of samples containing Mn^{2+} at low concentration. The line width shown is that of the fifth hyperfine line (from the low-field side of the spectrum) for all samples except Mn^{2+} in water and the fourth line for Mn^{2+} in water. The fifth line was chosen instead of the slightly narrower fourth line so as to avoid complications in the mitochondrial data arising from overlap of the hyperfine sextet with the strong spin exchange signal. For these experiments the mitochondria were prepared in the usual way. Mn^{2+} uptake for these samples was large (> 100 nmoles/mg protein). The Mn^{2+}–BSA–KCl sample contained approximately 30 % bovine serum albumin, 1 % KCl, pH 5.0. Glycerol concentration in the Mn^{2+} in water–glycerol sample was approximately 25 % by weight.

of line width on temperature for the bovine serum albumin solution and S are remarkably similar.

When the external osmolarity of the bathing solution for the mitochondria was halved, the line width of the fifth line of S decreased by 2.2 ± 0.7 Oe. Dilution of the bovine serum albumin solution by a factor of two resulted in a comparable reduction in line width, 3.6 ± 0.7 Oe.

These observations strongly support the hypothesis that the ≃ 30 Oe lines of S arise from a fraction of free Mn^{2+} with the dominant perturbation on line width being the viscosity of the concentrated protein solution within the organelles.

If this premise is accepted, it is a simple matter to estimate the concentration of free Mn^{2+} inside the mitochondria. Taking the free water within the inner membrane to be 0.4 μl/mg of protein at 0.25 M external osmolarity[10-12], it has been found in some experiments, where uptake was $>$ 100 nmoles Mn^{2+}/mg protein (as estimated from the sum of the intensities of the exchange *plus* hyperfine sextet spectra) that the free intramitochondrial Mn^{2+} concentration (as estimated from S alone) was greater than 10 mM. Since the concentration in the external medium was measured to be more than twenty times smaller, these results support the contention that divalent cations are indeed actively transported by mitochondria, *i.e.* they are translocated against an activity gradient.

When acetate is substituted for chloride in Ca^{2+} transport, the mitochondria swell and uptake is enhanced[13]. This has been interpreted to signify that the permeable anion acetate allows a greater amount of Ca^{2+} to exist in an osmotically active form. However, the replacement of chloride by acetate in our studies made little difference in the intensity of S or E, nor were the line shapes of either component measurably affected. The amounts of Mn^{2+} observed by EPR (S+E) with either chloride or acetate as the counterion were larger than the amount of Ca^{2+} transported where chloride was the counter ion[13] and roughly equivalent to the amount of Ca^{2+} transported when acetate was the counterion. It is usually maintained that cations alone are transported by active processes and that anion transport follows cation transport passively and independently except for charge balance requirements. Under such a model it is not understood how Mn^{2+} and Ca^{2+} uptake by mitochondria can differ when the accompanying anion is changed (*i.e.* if Ca^{2+} uptake is greater when acetate is present than when chloride is present then Mn^{2+} should behave similarly). It is possible that what has been observed here is a manifestation of some coupling between anion and cation accumulation in contradiction to the independent transport hypothesis. Evidence for such a coupling has been observed in K^+ uptake in mitochondria (G. Kimmich and H. Rasmussen, unpublished results).

The methods outlined in this paper may be applicable to a wide range of cellular and subcellular systems. Mn^{2+} as a paramagnetic analog of Ca^{2+} (or Mg^{2+}) can be incorporated into various biological systems and the levels of bound and free ions measured. Changes in these levels in response to treatment with various reagents, *e.g.* hormones, can be monitored. When the hyperfine sextet is present, the local internal viscosity or an upper limit to it can be estimated. Observed changes in viscosity, correlated with various treatments, can be used to follow phenomena such as swelling and contractile processes.

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