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Effects of chloride on paramagnetic coupling of manganese in calcium chloride-washed photosystem II preparations

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The effect of chloride on paramagnetic coupling of manganese in the oxygen-evolving complex of $CaCl_2$-washed PS II preparations was examined using Q-band ESR. When these PS II preparations were depleted of chloride, a strong 6-line ESR signal characteristic of protein-bound, uncoupled manganese was observed. Incubation at high chloride concentrations caused the disappearance of this signal. By repeated removal and addition of chloride, the signal could be cycled on and off without loss of bound manganese. When in a chloride-depleted state, the ESR-detectable protein-bound manganese could be removed by treatment with EDTA. Subsequent heating of EDTA-treated preparations revealed a second pool of protein-bound manganese associated with PS II. One of these pools requires a high concentration of chloride to maintain paramagnetic coupling while the second pool (within the limits of our observations) does not appear to require chloride for the maintenance of the paramagnetically coupled state.

Photosystem II *Photosynthesis* *ESR* *Manganese* *Cl^-* *Paramagnetic coupling*

1. INTRODUCTION

Numerous lines of evidence [1] suggest that manganese plays a central role in the oxidation of water in PS II. Treatments which release bound manganese such as alkaline Tris [2], hydroxylamine [3] or heat treatment [4], destroy the ability of photosynthetic membranes to evolve oxygen. There also exists an absolute requirement for Cl^- in the process of oxygen evolution [5]. The oxygen-evolving complex in Cl^--depleted membranes loses its ability to proceed past the Kok S_2 state [6,7]. Additionally, after Cl^- depletion, bound manganese becomes much more labile to alkaline Tris [8], hydroxylamine [9] and heat treatment [4]. When oxygen-evolving PS II membranes are washed with 1.0 M $CaCl_2$ the extrinsic polypeptides (33, 24 and 14 kDa) are lost from the membrane without the concomitant loss of bound manganese [15]. The 24 and 14 kDa extrinsic polypeptides appear to act as Cl^- concentrators [10,11]. PS II membranes which have had these polypeptides removed exhibit a high Cl^- requirement for the maintenance of bound manganese; at low Cl^- concentrations (<200 mM) bound manganese is lost. These results suggest that the functions of bound manganese and Cl^- are intimately entwined.

Here we shall examine the interaction of Cl^- with bound manganese in $CaCl_2$-washed PS II membranes. We have used Q-band ESR (35 GHz) to observe protein-bound, but non-paramagnetically coupled, manganese (see section 3) associated with these PS II membranes. Our results indicate that Cl^- is required for the maintenance of paramagnetic coupling within one pool of protein-bound manganese. This manganese pool can be cycled repeatedly between the paramagnetically coupled and uncoupled states by

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; PS II, photosystem II

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repeated addition and removal of Cl^-. Additionally, when this pool of manganese is in the paramagnetically uncoupled state, it can be removed from the photosynthetic membrane with EDTA. However, when this manganese is in a paramagnetically coupled state it is inaccessible to the chelating agent. We have also identified a second pool of paramagnetically coupled manganese which does not require Cl^- (at least under the conditions examined) for the maintenance of coupling.

2. MATERIALS AND METHODS

Chloroplasts were isolated from market spinach as described [12]. Oxygen-evolving PS II membrane preparations were prepared essentially according to the procedure of Ghanotakis and Babcock [13]. The chloroplasts were, however, incubated at 4°C for 1 h under stacking conditions [14] prior to detergent treatment and were washed with resuspension buffer (300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl and 50 mM Mes-NaOH, pH 6.0) after detergent treatment. This preparation exhibited no Signal I when oxidized with ferricyanide in the dark. The extrinsic polypeptides of the oxygen-evolving complex were removed by washing the PS II membranes with resuspension buffer containing 1.0 M $CaCl_2$ [15].

The polypeptide-depleted PS II membranes were washed twice with resuspension buffer, sampled for ESR spectroscopy, washed twice with resuspension buffer containing 2.0 M NaCl, and re-sampled for ESR spectroscopy. This cycle of treatment with low Cl^- (35 mM) and high Cl^- (2.0 M) resuspension buffer could be repeated at least 4 times. The amount of Cl^- required to induce the re-establishment of paramagnetic coupling in low Cl^--washed PS II membranes was determined by suspension and washing in resuspension buffer with varying amounts of Cl^- (50 mM to 2.0 M). Non-paramagnetically coupled manganese was removed from Cl^--depleted PS II membranes by washing with resuspension buffer containing 20 mM EDTA. These membranes were then washed twice with resuspension buffer and sampled for ESR spectroscopy. These samples were then heated to 100°C for 5 min in the ESR tube and new spectra were taken. During all of these treatments the PS II membranes were resuspended to 1.0 mg/ml Chl during washing and were incubated for 30 min at 4°C. Sample sizes for ESR spectroscopy were 10 μl at a Chl concentration of 10.0 mg/ml Chl.

Q-band ESR spectra were obtained on a 35 GHz Varian E 109 Q spectrometer at 90 K. Instrument conditions: microwave power 5 mW, microwave frequency 35.0 GHz, modulation amplitude 5.0 G, modulation frequency 100 kHz, scan rate 31.25 G/min, time constant 1 s.

3. RESULTS AND DISCUSSION

In aqueous solution, Mn(II) ions experience an octahedral crystal field when averaged over time. For this highly symmetrical environment, the ESR spectrum exhibits 6 equally spaced lines (95 G separation). Second order effects cause the linewidths to be somewhat field dependent, and each of the 6 lines has a slightly different width, but the spectrum is well resolved and has essentially the same appearance at both X-band (9 GHz) and Q-band (35 GHz) (fig.1a).

When Mn(II) exists in an anisotropic crystal field, as is the case when it is bound to protein (and not paramagnetically coupled), its ESR spectrum consists of 30 allowed transitions, 24 of which are extremely broad in a powder spectrum. The remaining 6 components (i.e. the $m_s = +1/2 \rightarrow -1/2$ transitions) are narrow, in some cases nar-

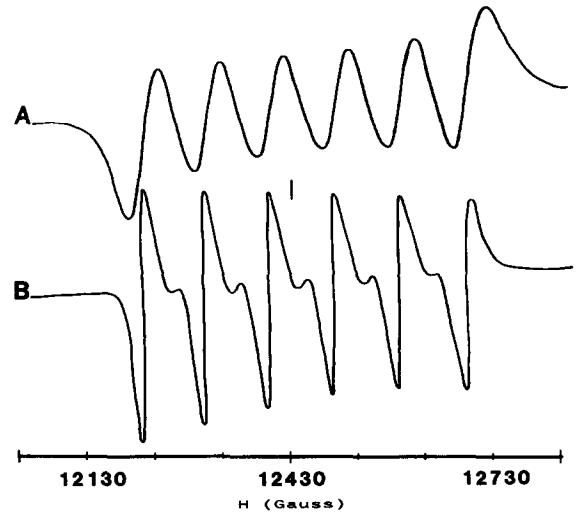

Fig.1. Q-band ESR spectra of (A) 30 mM $MnCl_2$ and (B) the protein-bound manganese signal of concanavalin redrawn from [17].

rower than the 6 lines of aqueous Mn(II) [16–18] (fig.1B).

Experimentally, there are three major differences between the spectra of isotropic and anisotropic Mn(II) [16–20].

(i) The intensity of the ESR powder spectra obtained from Mn(II) in an anisotropic environment is 9/35 as intense as that from an equal concentration of isotropic Mn(II).

(ii) The widths of the 6 major lines from anisotropic Mn(II) are strongly field dependent. At Q-band the widths are comparable to, or narrower than, those from the aqueous ion, while at X-band the widths are typically much greater. Under similar operating conditions, a Q-band spectrometer can detect well resolved Mn(II)-protein signals while an X-band instrument would find a broad, featureless spectrum.

(iii) 'Forbidden lines', double quantum transitions, appear in the intervals between the 6 major lines of anisotropic Mn(II). For example, small signals from forbidden lines can be seen in fig.1B. Their presence is definite evidence of an anisotropic crystal field environment for the Mn(II); forbidden lines are absent when the field is isotropic. Forbidden lines are much weaker at Q-band than they are at X-band where they clutter the spectrum and contribute to the loss of resolution.

Because of these differences, paramagnetically uncoupled Mn(II)-protein complexes are easily detected with Q-band ESR, but not with X-band instruments. In our studies we have exploited the advantages of Q-band ESR spectroscopy to investigate the effects of Cl^- on the paramagnetic coupling of Mn(II) associated with the oxygen-evolving site of PS II.

Fig.2 illustrates the results of a typical experiment in which oxygen-evolving PS II membrane preparations [13] (fig.2A) are first washed with 1.0 M $CaCl_2$ (fig.2B) and then resuspended in low chloride media (resuspension buffer, 45 mM Cl^-). Preparations treated in this manner exhibit a large, 6-line signal which is indicative of protein-bound, and paramagnetically uncoupled, Mn(II) (fig.2C). If such a preparation is resuspended in a high chloride medium (resuspension buffer + 2.0 M NaCl), the intensity of the protein-bound Mn(II) signal is greatly diminished (fig.2D). Fig.2E demonstrates that the reduction in intensity of the protein-bound Mn(II) signal is not due to the loss of Mn(II) from the membranes since resuspension of these membranes in low chloride media restores the protein-bound Mn(II) signal to its original high level. Indeed, membranes can be cycled repeatedly through these treatments (at least 4 times) without any apparent change in maximal signal intensity in the Cl^--depleted state. This strongly suggests that the decrease in protein-bound Mn(II) signal intensity in the presence of high Cl^- concentrations is due to the induction of paramagnetic coupling within the Mn clusters of PS II. Fig.3 illustrates the extent of paramagnetic coupling induced in

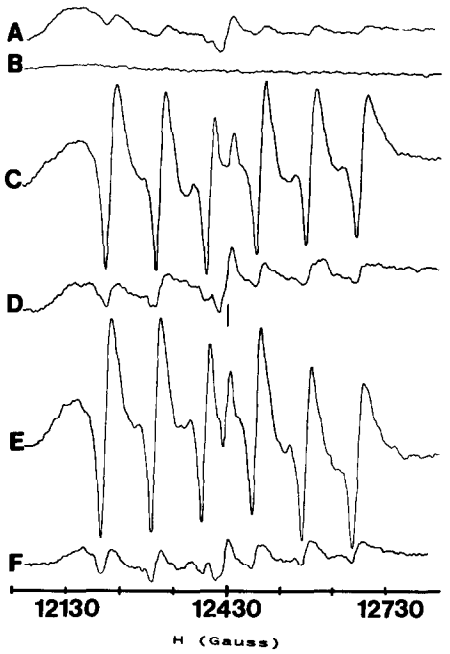

Fig.2. The effect of Cl^- depletion and re-addition on $CaCl_2$-washed PS II preparations. Q-band ESR spectra of: (A) untreated oxygen-evolving PS II membranes; (B) $CaCl_2$-washed PS II membranes; (C) sample (B) washed and suspended in resuspension buffer (35 mM Cl^-); (D) sample (C) washed and suspended in 2.0 M NaCl; (E) sample (D) washed and suspended in resuspension buffer (35 mM Cl^-); (F) sample (E) washed and suspended in 2.0 M NaCl. Spectrometer sensitivity was essentially constant during all 6 runs. The small amount of protein-bound manganese visible in (A) can be completely removed by treatment with EDTA with no effect on the results of the subsequent treatments.

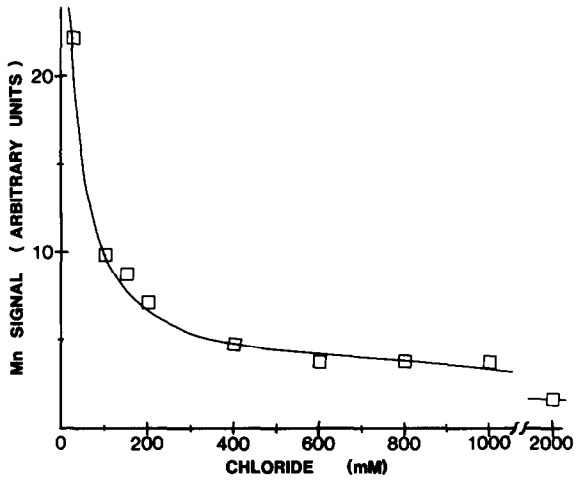

Fig.3. The effects of different concentrations of Cl^- on the relative signal intensity of protein-bound, paramagnetically uncoupled manganese. The manganese was uncoupled as in fig.2C and the membranes were resuspended in buffers containing NaCl concentrations ranging from 50 mM to 2.0 M.

$CaCl_2$-washed and Cl^--depleted PS II membranes upon resuspension in buffer containing varying concentrations of Cl^-. About 100 mM Cl^- is required to reduce the signal intensity of uncoupled protein-bound manganese by 50%.

Treatment of oxygen-evolving PS II membranes with 1.0 M $CaCl_2$ is known to release polypeptides with apparent molecular masses of 33, 24 and 17 kDa without the concomitant release of bound manganese [15]. Additionally, $CaCl_2$-washed membranes which are resuspended in low chloride media gradually lose bound manganese [21]. This loss can be prevented by the presence of high concentrations (200 mM) of Cl^-. Our results suggest that the increased lability of bound manganese in the presence of low Cl^- concentrations is associated with the uncoupling of the manganese within PS II.

The results shown in fig.4 demonstrate that the uncoupled, protein-bound manganese which is observed after Cl^- depletion (fig.4B) is accessible to the chelating agent EDTA (fig.4C). Protein-bound manganese which is paramagnetically coupled is not accessible to the chelating agent (not shown). If the membranes which have been depleted in Mn(II) by EDTA treatment are subsequently heated at 100°C for 5 min to denature the membrane proteins (after removal of EDTA), a

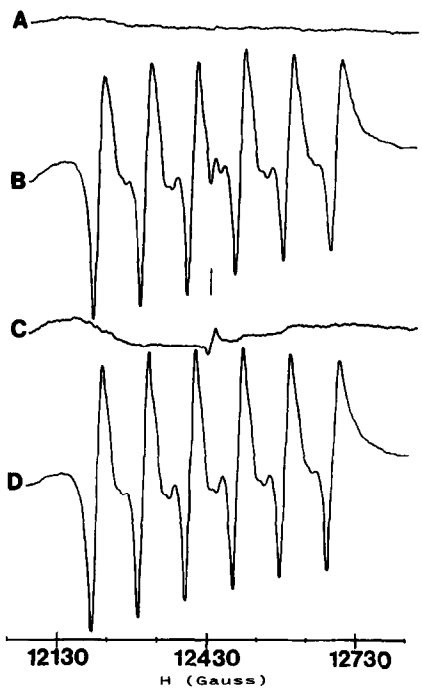

Fig.4. The identification of two pools of manganese in $CaCl_2$-washed PS II membrane preparations. Q-band ESR spectra of: (A) $CaCl_2$-washed PS II membranes; (B), (A) washed and suspended in resuspension buffer (35 mM Cl^-); (C), (B) after washing with 20 mM EDTA and removal of the EDTA by washing and suspension in resuspension buffer (35 mM Cl^-); (D), (C) heated to 100°C for 5 min.

second population of uncoupled protein-bound manganese appears (fig.4D). These data strongly suggest the existence of two pools of bound manganese associated with PS II. One pool requires a high concentration of Cl^- to maintain paramagnetic coupling in $CaCl_2$-washed membranes. The second pool remains paramagnetically coupled even at relatively low (50 mM) Cl^- concentrations. Paramagnetic coupling of the second manganese pool may be independent of Cl^-, or it may require Cl^-, but at lower concentrations than we have examined.

A number of workers have reported heterogeneity in the manganese pool associated with PS II. Yocum et al. [22] have shown that all of the manganese ions associated with PS II are not equivalently extracted with NH_2OH or Tris. Klimov et al. [23] have reported that donor reac-

tions in PS II (but not oxygen evolution) can be restored in manganese-depleted PS II preparations by the addition of two manganese ions per reaction center. Finally, in $CaCl_2$-washed PS II membranes, half of the manganese is unstably associated with the membrane during incubation at low Cl^- concentrations [24]. These appear to be the same manganese ions which are released by trypsin treatment of PS II membrane preparations [25]. Our findings are consistent with these results and help to provide a molecular rationale for these phenomena.

Two general models have been proposed for manganese-chloride interactions at the oxygen-evolving site of PS II. Our results are consistent with either model. Sandusky and Yocum [26] and Critchley and Sargeson [27] have suggested that Cl^- may act as a bridging ligand between manganese atoms. Coleman and Govindjee [28] have suggested a charge screening role for Cl^- at a site distant from the active site of oxygen evolution. Our results show that in $CaCl_2$-washed membranes a high concentration of Cl^- is required to maintain paramagnetic coupling within at least one population of the protein-bound manganese. Under the conditions examined, a second pool of manganese exists which does not require a high concentration of Cl^- to maintain paramagnetic coupling. This population of manganese becomes ESR-detectable after protein denaturation. We suggest that Cl^- depletion induces a conformational change in the protein(s) which interact with the bound manganese. This conformational change leads to a loss of paramagnetic coupling and an increased accessibility of one population of bound manganese to chelating agents such as EDTA.

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