A surface-enhanced Raman signal associated with functional manganese in oxygen-evolving photosystem II membranes

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A surface-enhanced Raman signal at about 225 cm^{-1} was detected in oxygen-evolving photosystem II (PS II) membranes treated with 1 M $CaCl_2$ (which removes three extrinsic proteins but does not extract Mn). The signal is not seen in Tris-treated preparations (which retain none of the three proteins nor Mn) nor in NaCl-treated preparations (which retain Mn and the 33-kDa extrinsic protein). The Raman signal is unstable and has been related kinetically, both to the disappearance of $CaCl_2$-reconstituted O_2 evolution and to the release of about half the Mn found initially in the $CaCl_2$-treated material. Although the origin of the signal may be either a direct or indirect monitor of the unstable Mn pool, the frequency is consistent with Mn-N, Mn-O or Mn-Cl vibrations reported in the literature. From these data and previous studies we suggest a model in which the unstable pool of Mn is located between the 33-kDa extrinsic protein and the PS II membrane, probably at the site of a 34-kDa intrinsic protein.

O_2 evolution *Manganese* *Photosystem II* *33-kDa protein* *34-kDa protein* *Photosynthesis*

1. INTRODUCTION

The availability over the past few years of various PS II preparations which retain the capacity to split water (see [1] and references therein) has greatly stimulated interest in the photosynthetic oxygen-evolution process. The fact that both the phase-partitioned vesicles [2] and the detergent-fractionated membrane preparations [1] are in the inside-out configuration with the oxygen-evolving complex located on the external (original lumen) surface has made them very useful for research purposes. Recent studies probing function on the oxidizing side of PS II have emphasized the extrinsic membrane proteins at about 17, 23, and 33 kDa purportedly associated with the oxygen-evolving enzyme complex [3–5]. There is also new evidence that a 34-kDa intrinsic protein, which is a component of the PS II core complex, is involved in the water-splitting process [6]. This protein is distinct from the 33-kDa extrinsic protein and from the herbicide-binding protein associated with the reducing side of PS II.

During the past year, interest has refocused on the location and function of manganese, long known as required for oxygen evolution [7]. Many procedures, including alkaline-Tris treatment, which removes the three extrinsic proteins, also release Mn from the membrane. However, $CaCl_2$ [8] and urea [9] recently were found to release these proteins without extracting functional manganese. Since the extracted PS II membrane must contain a Mn-binding site, probably on the 34-kDa intrin-

Abbreviations: Mes, 2-[*N*-morpholino]ethanesulfonic acid; OES II, oxygen-evolving photosystem II; PS II, photosystem II; SCE, standard calomel electrode; SERS, surface-enhanced Raman scattering

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sic protein [6,10], we have employed SERS spectroscopy to probe the surface of $CaCl_2$- and Tris-treated OES II membranes for a signal that could be attributed to Mn. This note reports the discovery of such a signal. Although the technique has been used successfully to study a variety of proteins, including cytochrome *c*, myoglobin [11], cytochrome cd_1 [12], reaction centers from photosynthetic bacteria [13], and glucose oxidase (T. Cotton, unpublished results), this is, to our knowledge, the first application of SERS to the study of a membrane system.

2. MATERIALS AND METHODS

OES II preparations were obtained from spinach chloroplasts using Triton X-100 [5]. These membranes are isolated from the appressed granal region of thylakoids. As appressed membrane fragments, both surfaces are lumenal sides, and contamination by PS I pigments is minimal as judged by EPR and gel electrophoresis studies [1]. Steady-state O_2-evolution rates (controls were 300–350 μmol $O_2 \cdot$ mg $Chl^{-1} \cdot h^{-1}$) were assayed using 1 mM 2,6-dimethylbenzoquinone (Aldrich, Milwaukee) as an acceptor in a buffer containing 20 mM Mes (pH 6.0), 15 mM NaCl, and 300 mM sucrose, unless noted otherwise. Two different treatments were used to remove the 17-, 23- and 33-kDa extrinsic proteins. Treatment A used 1 M $CaCl_2$ in 40 mM Mes-NaOH (pH 6.5), 10 mM NaCl, and 300 mM sucrose or sorbitol, which does not extract functional Mn from the membrane [8], and treatment B employed 960 mM Tris-HCl (pH 9.3) in 300 mM sucrose, which removes >85% of the Mn from the OES II membrane [14]. Treatment C (2 M NaCl, pH 6.0) which removes all of the 17- and 23-kDa extrinsic proteins while leaving the 33-kDa extrinsic protein and Mn intact, was made according to Sandusky et al. [15]. All treatments were performed at 0.5 or 1.0 mg/ml Chl for 30 min (4°C) in room light except that treatment A was not exposed to light; the samples were centrifuged at $30000 \times g$ for 15 min, and the pellets were washed, then resuspended in 20 mM Mes (pH 6.5), 15 mM NaCl, 5 mM $MgCl_2$ and 400 mM sucrose (medium A). OES II samples exposed to the three treatments were first washed in medium A containing 0.5 mM EDTA to remove non-specific Mn. All samples were kept at −70°C in medium A until use.

Surface-enhanced Raman spectra were obtained using the instrumentation and procedures described previously [11]. Samples were adsorbed onto a silver electrode following an anodization process. Anodization consisted of oxidation at 0.45 V vs SCE until 25 mC/cm^2 current passed, followed by reduction at −0.6 V vs SCE. Following anodization of the electrode in an electrolyte buffer consisting of 100 mM Na_2SO_4, 20 mM Mes (pH 6.0), and 300 mM sucrose, aliquots of treated OES II preparations were added to the experimental cell. Chloride ions were not used in the buffer because of interference due to a strong Ag-Cl vibration in the vicinity of the new low frequency mode observed in the OES II samples. The solution was N_2-purged prior to and during addition of sample to insure adequate mixing. An investigation of the effect of electrode potential on the signal intensity determined that maximum intensity was observed near −0.2 V vs SCE.

Raman spectra were excited using the 514.5 nm line of an Ar-ion laser (Coherent Radiation, Innova 90-5). Chlorophyll and carotenoid interference was not observed in the low frequency region of interest. Back-scattering geometry was used, and the scattered light was collected with a Canon f/1.2 lens. The light was focused onto the slits of a Spex Triplemate 1877 monochromator/spectrograph and dispersed by a 1200 groove/mm grating onto an intensified silicon photodiode array detector (Model 1420, EG&G PARC). Data collection and analysis were accomplished using an OMA II system (EG&G PARC).

3. RESULTS AND DISCUSSION

Preliminary attempts to detect a SERS signal attributable to the presence of Mn by comparing control and alkaline Tris-treated OES II preparations were not successful (not shown). However, when $CaCl_2$-treated (normal amount of membrane-bound Mn) and Tris-treated (little membrane-bound Mn) preparations were compared, a new signal was identified in the former. Fig.1 shows low frequency spectra of both preparations and their respective buffer controls. Note the peak at about 225 ± 5 cm^{-1} in the case of the $CaCl_2$-treated sample. Since both treatments release the

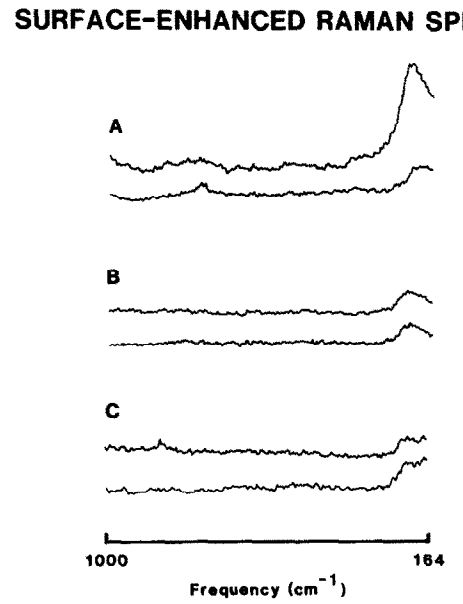

Fig.1. Low-frequency surface-enhanced Raman spectra of (A) $CaCl_2$-, (B) Tris- and (C) NaCl-treated OES II membranes along with their respective buffer blanks (lower spectrum in each case) at 22°C. Chl concentration, 11 μg/ml.

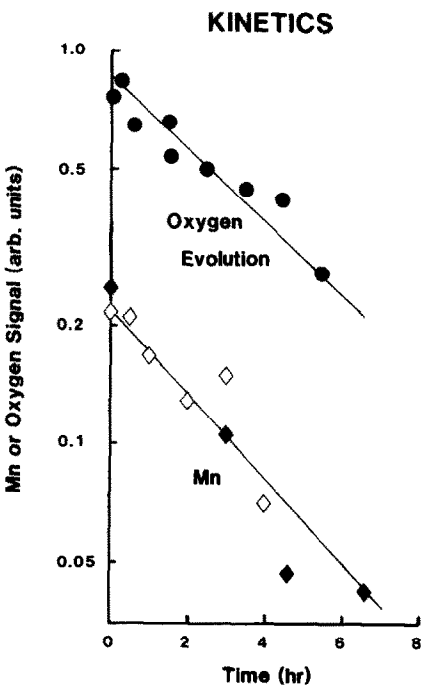

Fig.2. First-order plot of $CaCl_2$-activated O_2 evolution in $CaCl_2$-extracted OES II membranes (●——●). The samples were aged at ~0°C in the dark in a buffer containing 40 mM Mes (pH 6.5), 10 mM NaCl, and 300 mM sorbitol then assayed for O_2 in the same buffer. The second curve is a replot of data taken from fig.4 of [16] (◆——◆) and fig.1A of [9](◇——◇). It represents the release of Mn from the membrane under the same conditions used for aging the O_2 samples.

three extrinsic proteins, it appears that one or more of the three extrinsic proteins, when bound to the OES II membrane, can block detection of the new signal. The third set of spectra shown in fig.1 are of a NaCl-treated preparation which contains the 33-kDa extrinsic protein and the normal amount of membrane-bound Mn. Little evidence for a 225 cm^{-1} signal is apparent.

Ono and Inoue [16] found that about half the membrane-bound Mn was not stable in $CaCl_2$-treated OES II preparations. Furthermore, they reported that the readdition of 50 mM $CaCl_2$ to $CaCl_2$-treated samples restored up to 20% of the control OES II rate of O_2 evolution. In fig.2 we show that this $CaCl_2$-stimulated O_2 production can be used as a monitor of the Mn which falls off of $CaCl_2$-treated membranes. Note that the O_2-evolution capacity of the samples decreases with approximately the same kinetics as the release of the unstable Mn pool. Our samples were incubated at ~0°C in a buffer containing 40 mM Mes (pH 6.5), 10 mM NaCl, and 300 mM sorbitol. The Mn data are the labile component replotted from fig.4 of [16] (a $CaCl_2$-treated preparation) and fig.1A of [9] (a urea-treated preparation).

Fig.3 demonstrates that the new low frequency SERS signal is also unstable. However, it disappears much more rapidly than the Mn and O_2 results of fig.2 would indicate. Since the data in fig.3 were obtained in a buffer containing 100 mM Na_2SO_4 (instead of 10 mM NaCl) and at a temperature of 22°C (instead of ~0°C), we have plotted the disappearance of both the new Raman signal and $CaCl_2$-stimulated O_2 evolution under the conditions required to obtain the SERS signals. The results show that both the Raman and O_2 signals decrease with similar kinetics.

The fact that the new low frequency SERS signal reported here is observable only in OES II samples which contain Mn but lack all three extrinsic proteins suggests that the signal is related to Mn. It

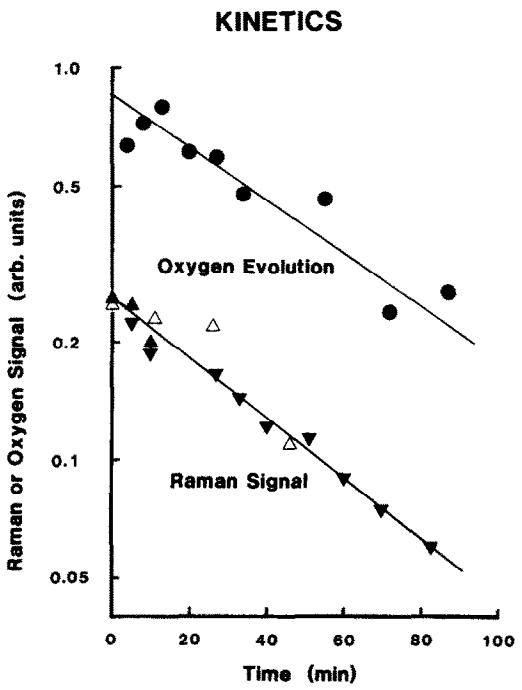

Fig.3. First-order plots of the disappearance of the surface-enhanced Raman signal (△, ▲, ▼) seen in fig.1A compared with the disappearance of $CaCl_2$-reconstituted O_2 evolution (●) obtained with similar samples aged under the same incubation conditions (i.e., 22°C in buffer containing 20 mM Mes (pH 6.0), 100 mM Na_2SO_4 and 300 mM sucrose). Chl concentration, 11 μg/ml (Raman); 5 μg/ml (O_2).

cannot be due to chlorophyll or carotenoids since both exhibit multiple Raman spectral lines below 1000 cm^{-1}, not observed in fig.1. That the signal is unstable and can be associated with the release of Mn from the membrane is further evidence supporting this conclusion. However, the exact origin of the SERS signal is uncertain. It is possible that the signal is a secondary effect and not a direct measure of the presence of Mn near the surface of the membrane. Nevertheless, it should be emphasized that there are low frequency modes in the vicinity of 225 cm^{-1} which have been identified with Mn-ligand vibrations. These include, for example, high spin Mn(II) Tris-bipyridine metal-to-nitrogen stretching vibrations [17]. The Cl^- complex of Mn(III) etioporphyrin also exhibits a band near 225 cm^{-1}. This has been attributed to a combination of Mn-Cl stretching motion together with motion of the metal atom against the porphyrin macrocycle [18]. Since Cl^- [19] and N or O [20] have been postulated as ligands to Mn, functional in the water-splitting process, the 225 cm^{-1} band in the SERS spectrum (fig.1) is consistent with a low frequency mode associated with Mn. (Fe-N vibrations have also been reported at about 220 cm^{-1} using 441.6 nm excitation [21], and OES II membranes contain iron. However, there is no evidence that iron falls off the membrane, so we would not expect to see a decrease of signal intensity with time, unless such an iron signal is itself an indirect probe of the unstable Mn pool.) If this is indeed true, then Mn must be close to the surface of our extrinsic protein-depleted OES II membranes.

Ono and Inoue [16] have suggested that there are two pools of membrane-bound Mn, one closer to the surface than the other. The evidence presented here is consistent with the idea that the Raman signal is a monitor of the surface pool which may be associated with the 34-kDa intrinsic protein [6,10]. The fact that little signal is seen in preparations that retain the 33-kDa extrinsic protein indicates that this protein covers the 'source' of the Raman signal. Abramowicz and Dismukes [22] have reported the isolation of the 33-kDa extrinsic protein with up to two Mn per protein by maintaining an oxidizing solution potential during isolation. We believe that the surface pool of Mn is located between the 33-kDa extrinsic and 34-kDa intrinsic proteins and that both proteins have Mn-binding sites as illustrated in fig.4.

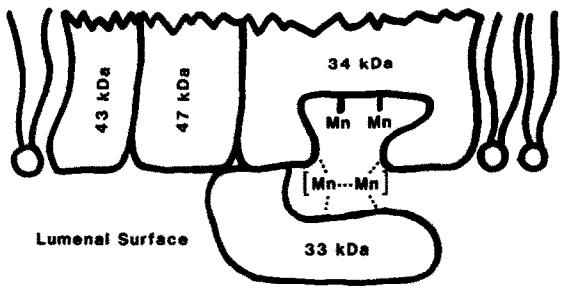

Fig.4. A model summarizing our ideas regarding the location of Mn in the PS II membrane. [Mn···Mn] represents the 'unstable' pool of functional Mn located between the 33-kDa extrinsic protein and the 34-kDa intrinsic protein. The dotted line represents uncertainty how the binuclear Mn complex is bound together. The other dotted lines indicate uncertainty how this Mn pool is bound to the two proteins. The other Mn in the model represents the stable membrane-bound pool.

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