Biochimica et Biophysica Acta, 592 (1980) 185—195

BBA 47889

FIELD-DISPERSION PROFILES OF THE PROTON SPIN-LATTICE RELAXATION RATE IN CHLOROPLAST SUSPENSIONS

EFFECT OF MANGANESE EXTRACTION BY EDTA, Tris, AND HYDROXYLAMINE

ROBERT R. SHARP [a] and C.F. YOCUM [a,b]

[a] *Department of Chemistry and* [b] *Division of Biological Sciences, The University of Michigan, Ann Arbor, MI 48109 (U.S.A.)*

(Received August 28th, 1979)
(Revised manuscript received March 17th, 1980)

Key words: Mn^{2+}; Hydroxylamine; NMR; Proton spin-lattice relaxation; Oxygen evolution; (Chloroplast)

Summary

Proton spin-lattice relaxation rates (R_1) have been measured in a variety of dark-adapted chloroplast suspensions over a range of field strengths between 1 and 15 kG (4—65 MHz). When the effects of EDTA or Tris washing on chloroplast relaxivities are compared, the pool of Mn associated with oxygen evolution is seen not to contribute significantly to relaxivity. Instead, nearly all of the observed relaxivity, which is characterized by a paramagnetic maximum near 20.7 MHz in the field dispersion profile of R_1, appears to arise from contaminating non-functional Mn(II) that can be removed by EDTA during the isolation procedure. These observations, which contradict previous reports ascribing chloroplast relaxivity to the water-oxidizing system, require a reevaluation of proposed models, derived from NMR studies, of the state of Mn in the water-splitting reaction.

Chloroplasts from which loosely bound non-functional Mn has been removed by EDTA washing do show an enhancement of relaxivity when exposed to NH_2OH at concentrations known to inactivate water oxidation. This NH_2OH-induced relaxivity is comprised of Mn(II) in two distinct paramagnetic sites. One site is chelatable by EDTA, whereas the other site is not. This finding suggests that some Mn(II) tightly bound to thylakoid membranes can contribute to relaxivity after inactivation of the oxygen-evolving reaction.

Abbreviations: PSII, Photosystem II; Chl, chlorophyll.

Introduction

It has recently been reported that the nuclear magnetic relaxation rates of water protons in suspensions of broken chloroplasts are significantly influenced by paramagnetic manganese in the thylakoid membrane [1—9]. The major portion of the total chloroplast manganese is closely associated with oxygen evolution and has been shown to lie on the oxidizing side of Photosystem II [10—12]. These manganese sites are quite labile, however, and attempts to isolate a mangano-protein complex that restores oxygen evolution activity have, at least until recently, met with limited success [13,14]. For this reason, a nonperturbing spectroscopic probe of bound manganese in native chloroplasts is of particular importance. Such a probe could in principle clarify a number of questions concerning the site of O_2 formation, such as the oxidation state(s) of manganese, the heterogeneity of the functional manganese pool, accessibility of water to the manganese hydration sphere, and redox changes in the water-splitting site produced by flash illumination sequences.

To further examine the role of membrane-bound manganese as a relaxation-producing agent, we have measured field-dispersion profiles and the time course of relaxivity changes that result from treatments with three manganese extractants. The agents used are (1) 1 mM EDTA, (2) 0.8 M Tris buffer, and (3) 5 mM NH_2OH. EDTA removes a variable portion of the total chloroplast manganese but causes little or no loss of oxygen evolution activity. Manganese extraction by 0.8 M Tris buffer eliminates oxygen activity almost entirely when conducted in light and concomitantly removes approximately sixty percent of the nonchelatable chloroplast manganese [15—21]. Hydroxylamine likewise extracts about sixty percent of the nonchelatable manganese and suppresses oxygen activity [15,21,23,24]. Unlike Tris, NH_2OH is fully effective at millimolar concentrations, it acts as an electron donor to Photosystem II of extracted chloroplasts [10,21,25], and its action is coupled to changes in the midpoint potential of cytochrome *b*-559 [21].

Comparison of the dispersion profiles resulting from these treatments shows that the functional manganese pool in dark-adapted chloroplasts has little, if any effect on the proton relaxation rate, at least in the absence of added reductants. Contaminating manganese, which can be removed without inhibiting either oxygen evolution activity or photophosphorylation, causes large relaxivity enhancements that may account entirely for previously observed effects. New enhancements relating to the very tightly bound (i.e., Tris- and NH_2OH-inextractable) manganese pool are also described.

Materials and Methods

Broken chloroplasts were prepared by the methods of Guikema and Yocum [26] or of Robinson and Yocum [27] where indicated. The chloroplasts were suspended in a buffer containing 400 mM sucrose, 20 mM Tricine (pH 8), 15 mM NaCl, 2 mM $MgCl_2$ and 0.2% bovine serum albumin (pH 8.0), distributed into glass vials and frozen at −70° C until use. After thawing, chloroplasts were used within 30 min at 25° C or 4 h at 3° C. Chloroplasts isolated in EDTA were prepared by the method of Robinson and Yocum,

except that 1 mM EDTA was present in the isolation buffer and chloroplasts were stored in the resuspending buffer without bovine serum albumin. To examine the effect of bovine serum albumin on proton relaxivity, chloroplasts in some experiments (as indicated) were pelleted and resuspended in bovine serum albumin-free buffer prior to measurement. As described below, bovine serum albumin had little effect on chloroplast relaxivity.

Oxygen evolution activity of washed thylakoids prepared by the method of Guikema and Yocum was inhibited by treatment with Tris or NH_2OH. For Tris inactivation, the broken chloroplasts were suspended in homogenizing buffer supplemented with Tris (0.8 M, pH 8.7 at 4°C) plus 1 mM EDTA, to a final concentration of 300 μg Chl/ml. These suspensions were stirred in an ice bucket in white light (100 000 ergs $\cdot$ $cm^{-2} \cdot s^{-1}$). Oxygen evolution activity was monitored as previously described [26]; exposure to Tris/EDTA for 20 min under the conditions described was sufficient to procedure >95% inactivation of oxygen evolution activity. Inhibition by NH_2OH plus EDTA was carried out as described by Ort and Izawa [28] and Horton and Croze [21]; washed thylakoids were resuspended in a buffer containing 0.2 M sucrose, 20 mM Tricine, pH 8, 2 mM $MgCl_2$ and 0.2% bovine serum albumin. Inhibition was initiated by adding neutralized NH_2OH to a final concentration of 5 mM and EDTA to a final concentration of 1 mM. The suspensions were stirred for 20 min in darkness. In all experiments described above, inactivated chloroplasts were harvested by centrifugation (10 000 $\times$ g, 10 min), washed twice in homogenizing buffer, and then resuspended in this buffer and frozen (—70°C) until assay. Experiments to monitor the kinetics of NH_2OH extraction by nmr were performed by adding 5 mM NH_2OH and 1 mM EDTA at the indicated times directly to sample tubes containing chloroplasts that had been isolated in EDTA.

Relaxation times were measured using a Bruker B/KR-322s (4—60 MHz) pulsed spectrometer and a Varian V-3401 electromagnet. The field was stabilized with a superstabilizer and locked on an external ^7Li resonance. T_1 values were measured using a modified triplet sequence $(180(o)\text{-}\tau_1\text{-}((90(o)\text{-}\tau_2\text{-}180(\pi)\text{-}\tau_2\text{-}90(o))\text{-}\tau_1)_n)$ in which signals were sampled following the $180(\pi)$ pulse using a gated integrator and stored in a Fabri-Tek Model 1064 Signal Averager. Data were transferred to a Commodore Model 2001 microcomputer for least-squares analysis. The microcomputer also provided precisely timed measurement sequences in kinetic experiments. The precision of T_1 measurements was ±3%; the absolute accuracy was checked by comparing measured T_1 values of degassed acidified water against literature values of T_1 obtained by the inversion-recovery method.

Theory

Manganese associated with Photosystem II appears to be located on the inner surface of the thylakoid membrane [16]. In order to monitor internal sites, it is important that the kinetics of water exchange across the membrane be rapid on the NMR time-scale. In a recent study we have shown that this condition is met in osmotically tight chloroplasts [29]. Consequently the spin-lattice relaxation rate, $R_1 \equiv T_1^{-1}$, of the water proton resonance is a weighted average

of relaxation rates in the internal and external media, and the proton NMR signal which results primarily from solvent in the supporting buffer, monitors relaxation-producing interactions in the internal (as well as external) aqueous environments.

Relaxation by paramagnetic ions has been discussed systematically elsewhere [30]. Nuclear relaxation is produced by magnetic dipolar and scalar interactions in a manner described by the Solomon-Bloembergen-Morgan (SBM) equations:

$$T_{1p}^{-1} = (q[\mathrm{Mn}]/[\mathrm{H_2O}])/(T_{1\mathrm{M}} + \tau_{\mathrm{M}}) + (T_{1p}^{-1})_{\mathrm{OS}} \tag{1}$$

$$T_{1\mathrm{M}}^{-1} = 7.2(10^{-32})\frac{S(S+1)\,g^2}{r^6}\left[\frac{3\tau_{\mathrm{C1}}}{1+\omega_{\mathrm{I}}^2\tau_{\mathrm{C1}}^2} + \frac{7\tau_{\mathrm{C2}}}{1+\omega_{\mathrm{S}}^2\tau_{\mathrm{C2}}^2}\right]$$

$$+ \tfrac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left[\frac{\tau_{\mathrm{e}2}}{1+\omega_{\mathrm{S}}^2\tau_{\mathrm{e}2}^2}\right] \tag{2}$$

where

$$\tau_{ei}^{-1} = \tau_{\mathrm{S}i}^{-1} + \tau_{\mathrm{M}}^{-1}\,, \quad \tau_{\mathrm{C}i}^{-1} = \tau_{\mathrm{S}i}^{-1} + \tau_{\mathrm{M}}^{-1} + \tau_{\mathrm{R}}^{-1}\,, \quad i = 1, 2$$

and

$$\tau_{\mathrm{S}i}^{-1} = \tau_{\mathrm{S,O}}^{-1}\left[\frac{0.2}{1+\omega_{\mathrm{S}}^2\tau_{\mathrm{v}}^2} + \frac{0.8}{1+4\omega_{\mathrm{S}}^2\tau_{\mathrm{v}}^2}\right] \tag{3}$$

$T_{1\mathrm{p}}^{-1}$ is the paramagnetic contribution to the relaxation rate when q molecules of water are coordinated to a paramagnetic ion of spin S and a specified g-value. In Eqns. 1 and 2, coordinated protons are assumed to be a distance r from the paramagnetic site, they have spin relaxation times equal to $T_{1\mathrm{M}}$ and $T_{2\mathrm{M}}$, their mean residence time prior to exchange with the bulk solvent is τ_{M}, the hyperfine coupling constant while on the metal is A, and $\hbar$ has its usual meaning. ω_{I} and $\omega_{\mathrm{S}} \cong 658\ \omega_{\mathrm{I}}$ are Larmor frequencies of the proton and electron spin respectively. τ_{R} is the rotational correlation time of the complex, $\tau_{\mathrm{S}1}$ and $\tau_{\mathrm{S},2}$ are relaxation times of the electron spin moment and $\tau_{\mathrm{S,O}}$ is the limiting value of $\tau_{\mathrm{S}i}$ when the magnetic field H_0 is zero. τ_{v} is the correlation time for fluctuations of the interaction between the spin moment and the ligand field. $\tau_{\mathrm{C}i}$ and τ_{ei} are, respectively, overall correlation times for the dipolar and hyperfine interactions. The 'outer sphere' contribution $(\mathrm{T}_{ip}^{-1})_{\mathrm{OS}}$, of bound paramagnetic ions to uncoordinated water protons has been described elsewhere [4,30].

Equations 1—3 predict a local maximum in R_1 when the following conditions are met: (1) $\tau_{\mathrm{R}}, \tau_{\mathrm{M}} > \tau_{\mathrm{S}i}$, (2) $2\omega_{\mathrm{S}}\tau_{\mathrm{v}} > 1$, and (3) $\omega_{\mathrm{I}}\tau_{\mathrm{S}i} \approx 1$. These conditions are characteristic of enzyme- or membrane-bound Mn(II) in typical laboratory magnetic fields (say 10 kG) [31—35] but not of higher oxidation states of manganese. They are also rarely satisfied by other first-row transition metal ions, with the possible exception of Cu(II). This ion, however, has a lower paramagnetic spin ($S = 1/2$) and, in consequence of Eqn. 2, a 35-fold lower molar relaxivity than Mn(II) for equal values of $\tau_{\mathrm{S}i}$. Thus a local maximum near 20 MHz in the dispersion profile of R_1 is direct evidence of a paramagnetic contribution to the relaxivity due to membrane-bound Mn(II)

or Cu(II) and is fairly specific evidence for involvement of the former. Since the observation of a paramagnetic maximum requires long reorientational correlation times (condition (1) above), this feature results only from macromolecular binding sites and not from hexaquo Mn(II) or from low molecular weight aqueous chelates.

Results

Proton relaxivity in chloroplast suspensions capable of oxygen evolution and photophosphorylation

The dispersion profiles and oxygen evolving and photophosphorylation activities of several chloroplast suspensions are summarized in Fig. 1 and Table I. An examination of Fig. 1 shows that isolation of chloroplasts in the absence of EDTA generates preparations of variable relaxivity, with pronounced paramagnetic maxima in the region between 20—30 MHz (circles). As indicated above, this feature is highly characteristic of Mn(II) bound in a site of low reorientational mobility, and we therefore interpret the data in Fig. 1 to indicate a major contribution to relaxivity by membrane-bound solvent-accessible Mn(II). Other dispersion profiles shown in Fig. 1 demonstrate the profound influence of EDTA on chloroplast relaxivity. For example, addition of EDTA (1 mM) to chloroplasts not previously exposed to the chelator serves to lower R_1 (triangles). An even more substantial reduction in R_1 is obtained with chloroplasts isolated in the presence of EDTA and then washed free of chelator (open diamonds), or with similar preparations to which

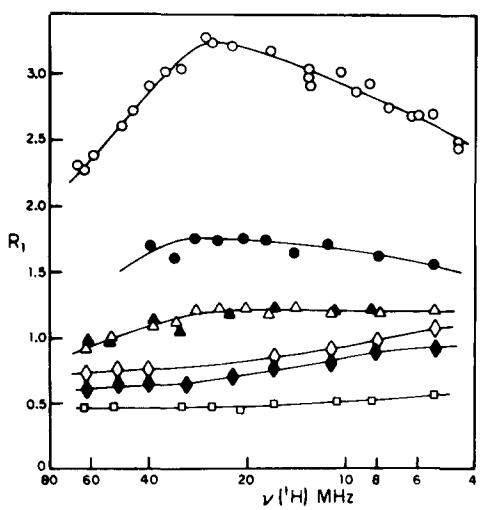


Fig. 1. Effect of EDTA on dispersion profiles of dark-adapted chloroplast suspensions (25°C). From top to bottom, profiles correspond to the following suspensions: ○———○, chloroplasts (2.5 mg Chl/ml), unexposed to EDTA; ●———●, identical preparation, using a different batch of leaves; △———△, suspension corresponding to open circles, after one wash in buffer containing 1 mM EDTA and resuspension in EDTA-free buffer; ▲———▲, suspension corresponding to closed circles after EDTA wash conducted as above; ◇———◇, chloroplasts (2.3 mg Chl/ml) isolated in 1 mM EDTA, resuspended in EDTA-free and bovine serum albumin-free buffer; ◆———◆, suspension corresponding to diamonds after addition of 1 mM EDTA; □———□, isolation buffer (identical with and without bovine serum albumin).

TABLE I

EFFECT OF EDTA ON PROTON RELAXIVITIES, OXYGEN EVOLUTION RATES, AND PHOTOPHOSPHORYLATION EFFICIENCIES OF DARK-ADAPTED CHLOROPLAST SUSPENSIONS

A. Isolation without EDTA; resuspension in presence or absence of EDTA. B. Isolation in 1 mM EDTA; resuspension in presence or absence of EDTA.

Chl, Chlorophyll concentration. The O_2 activity is presented as μmol O_2 per mg Chl per h (measured in the presence of 5 μg gramicidin, except BII, which was measured in the presence of 1 mM ADP and 5 mM P_i). The R_1 values are given in s^{-1} measured at 25°C, 20.7 MHz. $R_{1,cor} = (R_1 - R_1 \text{ (buffer)})/\text{Chl}$. ± EDTA indicates the presence or absence of 1 mM EDTA in the NMR sample.

Preparation		Chl (mg/ml)	O_2 activity	R_1	$R_{1,cor}$	$p/2e$
A I	(—EDTA)	3.17	237	3.84	1.06	
	(+EDTA)		239	1.44	0.30	
A II	(—EDTA)	2.80	370	1.98	0.54	
	(+EDTA)		372	1.28	0.29	
A III	(—EDTA)	2.12	355	1.16	0.32	
	(+EDTA)		364	0.93	0.21	
A IV	(—EDTA)	2.62	365	1.75	0.49	
	(+EDTA)		355	1.10	0.24	
B I	(—EDTA)	2.89	355	1.04	0.19	
	(+EDTA)		359	0.87	0.13	
B II	(—EDTA)	2.23	193 (phosphor-	0.98	0.23	1.16
	(+EDTA)		183 ylating)	0.70	0.10	1.17
B III	(—EDTA)	2.85	355	1.02	0.19	
	(+EDTA)		—	0.89	0.14	

EDTA is again added (closed diamonds). These latter conditions reduce the relaxivity to values near that of the buffer (squares).

Of paramount importance to these experiments is the question of whether EDTA affects the physiological activity of the chloroplast suspensions. Table I summarizes the results of the effects of EDTA on R_1 (measured at 20.7 MHz), oxygen evolution, and photophosphorylation in a number of chloroplast preparations. Inspection of these data show unequivocally that although exposure of chloroplast membranes to EDTA, during or after isolation, or during assay of activity, has no effect on oxygen evolution or ATP synthesis, this chelator without exception reduces dramatically the relaxivity of these preparations. It is of interest to note here that the greatest reductions in relaxivity, normalized for chlorophyll concentration and corrected for the buffer contribution ($R_{1,cor}$), are obtained for membranes isolated in the presence of EDTA. The presence of high levels of O_2 evolving activity in these low relaxivity preparations clearly demonstrates a lack of correlation between these parameters with regard to the water-splitting reaction, contrary to previous reports [14].

A comparison of chloroplast thylakoid and bovine serum albumin Mn(II) binding sites

The binding of Mn(II) to solvent-exposed, reorientationally restricted sites such as those that might be present in the water-splitting reaction should give

rise to a dispersion profile approximating that observed with a protein such as bovine serum albumin. At the chlorophyll concentrations employed in our experiments (2.5 mg/ml), the extractable manganese concentration is about 25 μM (assuming 4 Mn/PSII trap, and one trap per 400 Chl molecules) [15]. If this concentration of Mn(II) is added to Tricine buffer, which chelates Mn(II) [36], only a slight increase in R_1 (0.06 s^{-1}) is observed as shown in Fig. 2, owing to the short reorientational correlation time of the Tricine-Mn(II) complex. If 30 μM bovine serum albumin is present along with the Tricine buffer, then a greatly augmented molar relaxivity is observed. As shown in Fig. 2, the resulting dispersion profile shows a paramagnetic maximum at 30 MHz (open circles) which is shifted to lower field (closed triangles) at lower temperature (3°C), consistent with Eqns. 1—3 for τ_{Si} in a field-dependent region. Similar results have been observed for other Mn(II)-protein complexes [31—35].

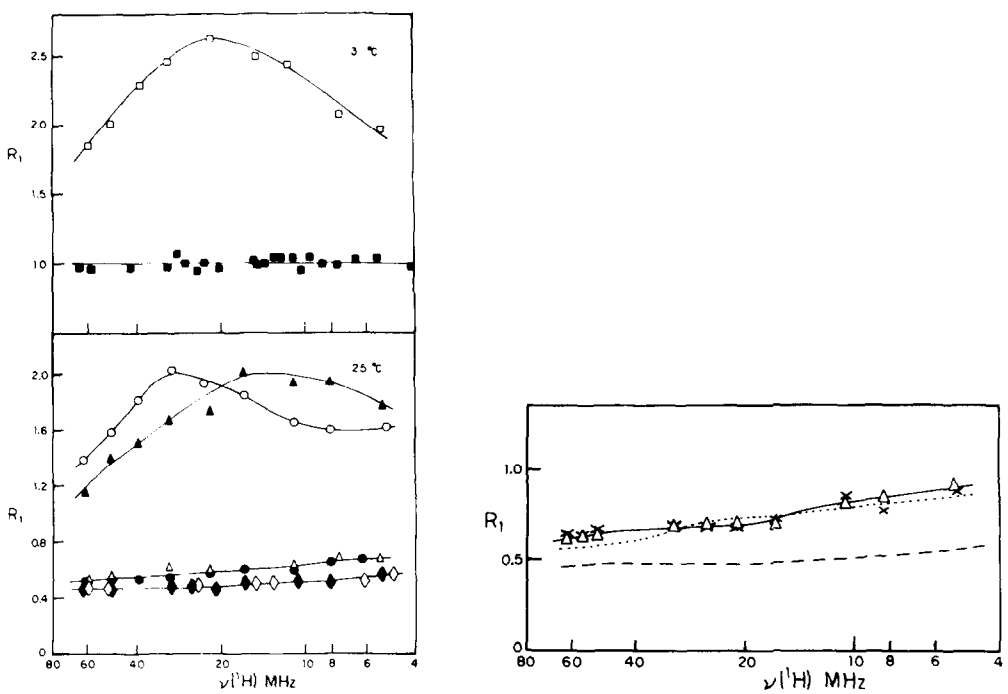


Fig. 2. Effect of added $MnCl_2$ and EDTA on dispersion profiles of chloroplasts and bovine serum albumin. From top to bottom, profiles correspond to the following solutions (data at 25°C except where indicated): □——□, isolation buffer containing 30 μM bovine serum albumin and 25 μM $MnCl_2$ (3°C); ■——■, resuspending buffer plus 30 μM bovine serum albumin (3°C); ▲——▲, chloroplasts (2.3 mg Chl/ml) isolated in EDTA and resuspended in EDTA-free buffer containing 25 μM $MnCl_2$; ○——○, suspension corresponding to (□) at 25°C; ●——●, resuspending buffer containing 25 μM $MnCl_2$; △——△, resuspending buffer plus 30 μM bovine serum albumin, 25 μM $MnCl_2$ and 1 mM EDTA; ◆, ◇, resuspending buffer with (◇——◇) and without (◆——◆) 30 μM bovine serum albumin.

Fig. 3. Effect of Tris extraction on the dispersion profile of dark-adapted chloroplasts (25°C). X——X, Tris/EDTA extracted chloroplasts (2.18 mg Chl/ml) resuspended in buffer containing 1 mM EDTA; △——△, chloroplasts (2.3 mg Chl/ml) isolated and resuspended in 1 mM EDTA; · · · · · ·, data of Wydrzynski et al. [4] for Tris/acetate extracted chloroplasts; - - - - - -, resuspending buffer.

The data in Fig. 2 provide an approximation of the response expected for solvent-accessible Mn(II) bound to reorientationally restricted protein sites in the presence of Tricine buffer, and one might expect to obtain results similar to these if functional Mn(II) in the water oxidizing complex were liganded in a similar manner. Fig. 2 also shows the dispersion profile for chloroplast membranes to which Mn(II), 25 μM final concentration, has intentionally been added. Mn(II) binds to the membranes [20], producing a dispersion profile with a maximum at a somewhat lower frequency than is observed for the Mn(II)-bovine serum albumin complex. Fig. 2 shows that addition of EDTA to either Mn(II) complex (chloroplasts or bovine serum albumin) reduces substantially the relaxivities of these systems.

It is of interest to note here that we have examined the abilities of both bovine serum albumin and carboxymethyl-cellulose to compete for Mn(II) bound to chloroplast membranes. Neither compound (data not shown) is capable of removing Mn(II) from its non-functional binding site on the thylakoid membrane, suggesting that this site is of limited accessibility to macromolecules even though it is readily accessible to EDTA.

Effects of Tris extraction

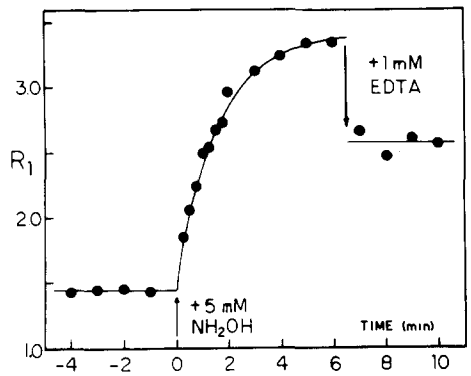
High concentrations of Tris buffer release approximately two-thirds of the tightly-bound chloroplast Mn with a concomitant loss of oxygen evolution activity and the appearance of a characteristic six-line hyperfine ESR spectrum of hexaquo Mn(II), which is apparently present as the ion in the thylakoid lumen [20]. The mechanism of Mn(II) release by Tris is unclear [15,16,20,22] although recent studies by Cheniae and Martin [22] indicate that illumination, which produces higher S-states (S_2 or S_3), sensitizes the water-splitting system to Tris inactivation.

Dispersion profiles of chloroplasts inactivated in the light in Tris (0.8 M) plus EDTA (1 mM) are shown in Fig. 3. This procedure, which caused a complete inactivation of oxygen evolution activity, produced chloroplast suspensions whose dispersion profiles differ very little from fully active O_2-evolving chloroplasts prepared in EDTA (Fig. 3). This result reinforces the conclusion that any functional Mn associated with the water-splitting complex is not monitored by NMR measurements.

Wydrzynski et al. [4] used Tris-acetone treatment to deplete Mn from chloroplasts. Their results, obtained on suspensions of somewhat higher chlorophyll concentration (3.0 mg/ml) than ours, are shown for purposes of comparison, as a dotted line in Fig. 3. It is evident that the two procedures (Tris plus acetone or EDTA) produce very similar end results, which in turn are very similar to those observed in fully functional chloroplasts isolated in EDTA.

Effects of NH_2OH extraction

When added to chloroplasts at concentrations ranging from 1 to 5 mM, NH_2OH extracts approximately two-thirds of the tightly-bound Mn [15,21,24]. This treatment, carried out either in the light or dark, produces a rapid inactivation of O_2 evolution [24]. Thus, NH_2OH extracts a functional Mn pool which appears to be similar to that liberated by Tris. Addition of Mn(II) to Tris-



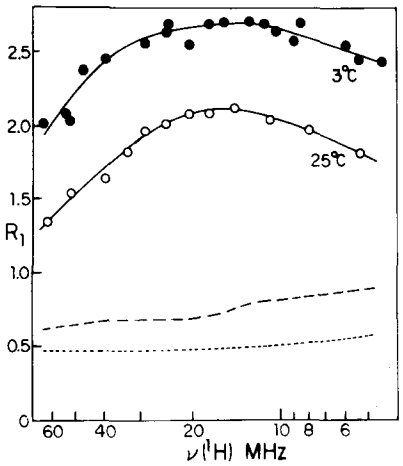


Fig. 4. Time course of proton relaxivity (20.7 MHz) during extraction by 5 mM hydroxylamine in the dark (pH 8.0, 3°C, 2.8 mg Chl/ml).

Fig. 5. Dispersion profiles of NH_2OH/EDTA extracted chloroplasts (2.5 mg Chl/ml) at 3°C (●——●) and 25°C (○——○); profiles for the resuspending buffer are shown for comparison.

washed chloroplasts [16,37] or whole algal cells treated with NH_2OH [24,38] serves to restore O_2 evolving activity, and this and other parallels [15] suggest that Tris and NH_2OH act to remove the same pool of Mn functional in oxygen evolution activity.

Fig. 4. shows changes in the proton relaxivity that occur during exposure of chloroplasts to 5 mM NH_2OH in the dark at 3°C. R_1 increases by a factor of more than two within the first four minutes after addition of NH_2OH. This time course closely parallels that of manganese loss and decay of oxygen evolution activity as measured by Cheniae and Martin [24] under similar conditions of extraction. Addition of EDTA to a final concentration of 1 mM at various times after the initiation of extraction suppresses a portion, approx. 45%, of the enhanced relaxivity. The elapsed interval between the addition of hydroxylamine and that of EDTA has very little influence on the EDTA-insensitive portion of relaxivity. Clearly, two classes of paramagnetic sites, which may be distinguished on the basis of their accessibility to EDTA-chelation, are exposed by NH_2OH.

Dispersion profiles of chloroplasts extracted in 5 mM NH_2OH/1 mM EDTA and resuspended in EDTA-free buffer are shown in Fig. 5. A pronounced paramagnetic maximum occurs near 14 MHz. The maximum is shifted to slightly higher frequency at 25°C. These profiles, which reflect the EDTA-insensitive portion of the enhancement, indicate that membrane-bound Mn(II) is responsible for the effect. The ESR spectrum of this preparation showed no six-line hyperfine pattern in these preparations, indicating the absence of free hexaquo Mn(II) (data not shown).

Discussion

Table I and Fig. 1 demonstrate conclusively that an EDTA-chelatable paramagnetic ion which is nonfunctional in O_2 evolution activity is the primary

source of relaxivity in suspensions of isolated chloroplasts. The variation in relaxivity observed with chloroplasts isolated in the absence of EDTA is probably a reflection of the variability, with season, of the leaf content of paramagnetic ions. Among such ions, Mn(II) is the most likely candidate as the contaminant, since dispersion profiles (Fig. 2) of chloroplast- and protein-Mn(II) complexes show maxima similar to those observed with EDTA-free chloroplasts.

By the simple expedient of including EDTA in various stages of treatment to suppress spurious signals from membrane-bound non-functional Mn(II), we have been able to show that Tris extraction (Fig. 3) does not generate an NMR-detectable signal in inactivated chloroplasts. Such a finding would agree with the results of Sauer and his colleagues [16,20] who detected a six-line hyperfine ESR signal characteristic of free hexaquo Mn(II) in Tris-washed chloroplasts; such a species would escape detection in the NMR experiments reported here.

Treatment of functional chloroplasts with NH_2OH (Figs. 4 and 5) creates a very different situation from that observed with Tris. The former agent, at concentrations know to produce inactivation of O_2 evolution (5 mM) produces a dramatic time-dependent rise in R_1, indicative of the appearance of Mn(II) bound to solvent-accessible, reorientationally restricted sites. Since the chloroplasts used for these experiments were isolated in EDTA, the Mn(II) seen here must originate from the functional pool of the ion associated with O_2 evolving activity. Furthermore, as shown in Fig. 4, two Mn(II) sites are involved. One of these sites is clearly accesssible to EDTA, whereas the other is not. The observation by Blankenship and Sauer [20] that EDTA can permeate the thylakoid membrane indicates that the EDTA accessible Mn(II) created by NH_2OH exposure may reside in the thylakoid lumen, near its site of release. By the same argument, the EDTA-inaccessible Mn(II) must reside in a special evironment which prevents the formation of the hexadentate EDTA-Mn(II) chelate, and that this site has a high retentiveness for the Mn(II).

A number of significant questions remain concerning the hydroxylamine effect we describe here. First, it is not known whether NH_2OH reduces solvent-exposed manganese to Mn(II) from some higher oxidation state or simply exposes to the solvent a previously sequestered Mn(II) site without reduction. However, NH_2OH is a highly efficient donor to PSII in extracted chloroplasts [21], and there is some evidence that such donation proceeds through the inextractable manganese pool [21]. Secondly, it is known that NH_2OH treatment in the dark leaves two inextractable manganese ions per PSII trap. Whether the observed relaxivity enhancement results from one or both of these ions remains to be determined. Thirdly, it is unknown whether the exposed Mn(II) is on the internal or external membrane surface.

In conclusion, these data and other investigations [39] leave little doubt as to the total lack of correlation between the relaxivities of isolated chloroplast suspensions and O_2 evolving activity. The present findings suggest that in its native form, the water-splitting complex contains Mn either in higher oxidation states (for example, Mn(III), or liganded in such a way as to prevent rapid exchange between the ion and the solvent. When spurious contributions to R_1 by loosely bound, nonfunctional Mn(II) are removed by EDTA washing, the

NMR technique is capable of detecting the appearance of new Mn(II) sites generated by the exposure of chloroplasts to NH_2OH. Further characterization of these Mn(II) sites is now in progress.

Acknowledgements

Mr. H.H. Robinson provided valuable assistance in preparing and assaying chloroplast suspensions. This research was supported in part by a grant (PCM 78-7909) to C.F.Y. from the National Science Foundation.

References

1 Wydrzynski, T., Zumbulyadis, N., Schmidt, P.G. and Govindjee (1975) Biochim. Biophys. Acta 408, 349—354
2 Wydrzynski, T., Zumbulyadis, N., Schmidt, P.G., Gutowsky, H.S. and Govindjee (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1196—1198
3 Wydrzynski, Y., Govindjee, Zumbalyadis, N., Schmidt, P.G. and Gutowsky, H.S. (1976) Am. Chem. Soc. Symp. Ser. 34, 471—482
4 Wydrzynski, T.J., Marks, S.B., Schmidt, P.G., Govindjee and Gutowsky, H.S. (1978) Biochemistry 17, 2155—2162
5 Govindjee, Wydrzynski, T. and Marks, S.B. (1978) in Photosynthetic O_2 Evolution (Metzner, H., ed.), pp. 321—324, Academic Press, New York, NY
6 Govindjee, Wydrzynski, T. and Marks, S.B. (1977) in Bioenergetics of Membranes (Papageorgiou, G. and Trebst, A., eds.), pp. 305—316, Elsevier/North Holland Biomedical Press, Amsterdam
7 Marks, S.B., Wydrzynski, T., Govindjee, Schmidt, P.G. and Gutowsky, H.S. (1978) in Biomolecular Structure and Function (Ayris, P.F., ed.), pp. 95—100, Academic Press, New York, NY
8 Gribova, Z.P., Zakharova, N.I. and Murza, L.I. (1978) Sov. Mol. Biol. 12, 157—164
9 Goldfeld, M.G., Vozvyshaeva, L.V. and Yushmanov, V.E. (1979) Biofizika 24, 264—269
10 Heath, R.L. (1973) Int. Rev. Cytol. 34, 49—101
11 Cheniae, G.M. (1970) Annu. Rev. Plant Physiol. 21, 467—493
12 Itoh, M., Katsuji, Y., Nishi, T., Katsuko, K. and Kazuo, S. (1969) Biochim. Biophys. Acta 189, 509—519
13 Tel-Or, E. and Avron, M. (1974) in Proc. 3rd Int. Congr. Photosynthesis, Rehovot, Israel, pp. 569—578, Elsevier, Amsterdam
14 Lagoutte, B. and Duranton, J. (1975) FEBS Lett. 51, 21—24
15 Cheniae, G.M. and Martin, I.F. (1970) Biochim. Biophys. Acta 197, 219—239
16 Blankenship, R.E., Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 387, 165—175
17 Yamashita, T., Tsuji, T., Yoshio, Y. and Giiti, T. (1972) Plant Cell Physiol. 13, 353—364
18 Yamashita, T. and Butler, W.L. (1968) Plant Physiol. 43, 1978-1986; Plant Physiol. 44, 435—438
19 Yamashita, T., Junko, T. and Giiti, T. (1971) Plant Cell Physiol. 12, 117—126
20 Blankenship, R.E. and Sauer, K. (1974) Biochim. Biophys. Acta 357, 252—266
21 Horton, P. and Croze, E. (1977) Biochim. Biophys. Acta 462, 86—101
22 Cheniae, G.M. and Martin, I.F. (1978) Biochim. Biophys. Acta 502, 321—344
23 Izawa, S., Heath, R.L. and Hind, G. (1969) Biochim. Biophys. Acta 180, 388—398
24 Cheniae, G.M. and Martin, I.F. (1971) Plant Physiol. 47, 568—575
25 Heath, R.L. and Hind, G. (1969) Biochim. Biophys. Acta 189, 222—233
26 Guikema, J.A. and Yocum, C.F. (1976) Biochemistry 15, 362—367
27 Robinson, H.H. and Yocum, C.F. (1980) Biochim. Biophys. Acta 590, 97—106
28 Ort, D.R. and Izawa, S. (1973) Plant Physiol. 52, 595—600
29 Sharp, R.R. and Yocum, C.F. (1980) Biochim. Biophys. Acta 592, 169—184
30 Dwek, R.A. (1975) Nuclear Magnetic Resonance in Biochemistry, Chapters 9—11, Oxford University Press, Oxford
31 Koenig, S.H. and Schillinger, W.E. (1969) J. Biol. Chem. 244, 6520—6526
32 Koenig, S.H., Brown, R.D. and Brewer, C.E. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 475—479
33 Brown, R.D., Brewer, C.F. and Koenig, S.H. (1977) Biochemistry 16, 3883—3896
34 Navon, G. (1970) Chem. Phys. Lett. 7, 390—394
35 Reuben, J. and Cohn, M. (1970) Adv. Enzymol. 33, 1—70
36 Good, N.E. and Izawa, S. (1972) in Methods in Enzymology (San Pietro, A., ed.), pp. 53—68, Academic Press, New York
37 Yamashita, T. and Tomita, G. (1974) Plant Cell Physiol. 15, 69—82
38 Radmer, R. and Cheniae, G.M. (1971) Biochim. Biophys. Acta 253, 182—186
39 Robinson, H.H., Sharp, R.R. and Yocum, C.F. (1980) Biochem. Biophys. Res. Commun. 93, 755—761