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On the interpretation of the NMR water-proton relaxivity of photosynthetic membrane samples: ramifications in the use of EDTA

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In thylakoid membrane samples which have had extraneous, nonfunctionally bound manganese ions removed by a low-osmotic wash medium, subsequent treatment with EDTA induces an additional loss in the proton-relaxivity enhancement of the sample. This EDTA-induced loss in proton-relaxivity enhancement occurs at a half-maximal concentration of about 30 μM EDTA for the spin–lattice (T_1) relaxation and about 100 μM EDTA for the spin–spin (T_2) relaxation. It is slowly time dependent, with a half-time of about 5 min, but it can be kinetically separated from the chelation function of EDTA which takes place in less than 3 min. This effect of EDTA can be reversed by thorough washing of the sample in buffer medium. In addition, treatment with EDTA reduces or masks the tetraphenylboron-induced increase in the proton relaxivity, which can also be reversed by thorough washing of the sample in buffer medium. Temperature-dependence measurements of the proton relaxivity at low-field proton-resonance frequencies indicate that the samples in the presence of EDTA do not reach the diamagnetic limit defined by the removal of functionally bound manganese. These results are interpreted to arise from EDTA-induced microenvironment changes in the membrane which restricts the accessibility of internally bound paramagnetic sites to the solvent phase protons and alters the spin exchange rate within the NMR time frame. It is suggested that this phenomenon may explain the failure to observe a correlation between the proton relaxivity, functionally bound manganese and the O_2-evolving capacity in samples treated with chelating agents (Sharp, R.R. and Yocum, C.F. (1983) Photobiochem. Photobiophys. 5, 193–199). As a consequence of the complex interaction of EDTA with the membrane, the importance and ramifications in the use of chelating agents in photosynthetic manganese measurements is therefore emphasized.

Introduction

It is without question that manganese plays a critical role in the O_2-evolving reactions in higher-plant photosynthesis [1,2]. However, unequivocal data on the exact functional nature of Mn is still lacking in the literature. There are at present five basic experimental approaches which have been used to probe Mn in photosynthetic membrane samples: (a) Mn X-ray absorption spectroscopy [3]; (b) a low-temperature multiline Mn ESR signal [4]; (c) a room-temperature six line hyperfine Mn ESR signal [5]; (d) an absorption change with a broad band peaking around 300–320 nm [6,7]; and (e) NMR water-proton

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Abbreviations: Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HTA^+, hexadecyltrimethylammonium; Mes, 4-morpholinepropanesulfonic acid; PS, Photosystem; TPB^-, tetraphenylboron.

relaxivity [8]. Each of these experimental approaches has advantages and disadvantages and is limited by the extreme complex nature of the photosynthetic membrane.

There is a long standing controversy in the literature as to whether the NMR water-proton relaxivity measurements reflect contributions only from extraneous, non-functionally bound Mn [9–14] or whether the proton relaxivity also contains contributions from the functionally bound Mn correlated with the O_2 activity as well [15–21]. The difference in the interpretations between the two groups lies basically in sample preparation and on the use of chelators, in particular EDTA. In Refs. 9–14 the basic assumption is made that the only effect of EDTA on the proton relaxivity of photosynthetic membrane samples is through the removal of extraneous, non-functionally bound Mn ions. In this communication we report a series of experiments on photosynthetic membrane samples which have had the extraneously bound Mn ions removed by a low osmotic wash medium (based on measurements of the total Mn content of the samples). Under this condition the NMR proton relaxivity displays a complex behavior in response to EDTA which cannot be explained by a simple chelation effect. Consequently, the importance and ramifications of experimental conditions, and in particular on the use of chelators, in the interpretation of proton relaxivity data are emphasized.

Experimental

Thylakoid membrane samples were isolated from 12–16-day-old pea plants grown under controlled conditions as described in Ref. 18. Washed leaves were homogenized in a Waring blender for approx. 15 s in a standard buffer medium consisting of 50 mM Hepes (pH 7.5)/400 mM sucrose/10 mM NaCl/5 mM $MgCl_2$. During homogenization 0.5% bovine serum albumin and 10 mM sodium ascorbate was also included in the medium. The homogenate was filtered through four layers of cheesecloth and a 10 μm mesh nylon cloth, centrifuged at $500 \times g$ for 1 min to remove cell debris, and again centrifuged at $2000 \times g$ for 10 min to collect the thylakoid membranes. The samples were then subjected to a wash at 200 μg Chl/ml (and allowed to equilibrate for approx. 5 min) with either the standard buffer medium or with a low osmotic buffer medium, in which case the standard buffer medium without sucrose was used. For some experiments, samples were also subjected to a Tris-acetone wash. In this case, samples that had been subjected to a low osmotic wash were suspended in a medium consisting of 0.8 M Tris (pH 8.3) and 20% acetone at a concentration of 200 μg Chl/ml for 20 min on ice in dim light. After the wash steps, the samples were finally suspended in the standard buffer medium containing sucrose and adjusted to 3 mg Chl/ml. EDTA was used as described in the text. Freshly prepared samples were used in all measurements of thylakoids.

Photosystem II (PS II)-enriched membrane fragments were prepared from greenhouse-grown spinach. Thylakoid samples were prepared as described above, except that the homogenization medium consisted of 20 mM Tricine (pH 8.0)/400 mM NaCl/2 mM $MgCl_2$/0.2% bovine serum albumin, and the wash medium consisted of 20 mM Tricine (pH 8.0)/150 mM NaCl/5 mM $MgCl_2$/0.2% bovine serum albumin. The thylakoid samples were then suspended in a buffer medium consisting of 10 mM Mes (pH 6.5)/400 mM sucrose/15 mM NaCl/4 mM $MgCl_2$, and treated with Triton X-100 in a ratio with the chlorophyll of 20 : 1 at a sample concentration of 2 mg Chl/ml for 15 min on ice in the dark with gentle stirring. The sample was then centrifuged at $35\,000 \times g$ for 20 min. The pellet was resuspended in the pH 6.5 buffer medium without detergent at a concentration of about 1 mg Chl/ml, and centrifuged at $5000 \times g$ for 10 min to remove unsolubilized membranes. The supernatant from this fraction was then centrifuged at $35\,000 \times g$ for 20 min to collect the PS II-enriched membrane fragments which were finally resuspended to approx. 4 mg Chl/ml in the pH 6.5 buffer medium containing 30% glycerol and stored at $-80°C$ until used. Before use, the samples were washed twice at 200 μg Chl/ml in the pH 6.5 buffer medium to remove the glycerol.

Steady state O_2-evolution measurements were made with a standard Clark electrode (Pt–Ag | AgCl) and a model 53 Yellow Spring monitor. Saturating illumination was obtained from a

tungesten lamp light source and was passed through a Corning CS 3-70 yellow filter and a two inch water filter containing 0.2% $CuSO_4$. Incident flux was approx. 250 mW/cm^2. The temperature during the measurement was maintained at 24°C by a temperature-controlled water circulator. The assay medium for thylakoid samples consisted of the standard buffer medium containing 2 mM $K_3Fe(CN)_6$ and 0.5 mM 2,6-dichlorobenzoquinone as the electron acceptors and 1 μM gramicidin D as an uncoupler. The assay medium for the PS II-enriched membrane samples consisted of the pH 6.5 buffer medium and the same concentration of electron acceptors used in the assay for the thylakoid samples. The sample concentration was 30 μg Chl/ml for these measurements.

The NMR proton spin–lattice (T_1) and spin–spin (T_2) relaxation at the low proton resonance frequencies were measured by the inversion recovery and Carr-Purcell (Meiboom-Gill modified) pulse sequences, respectively (eg., see Ref. 18). For the measurements at 90 MHz proton resonance frequency, the T_1 relaxation was measured by the inversion recovery method employing a composite pulse sequence [22,23]. All reported rates were corrected by subtracting the contribution from the buffer medium, plus any added reagents. All measurements were made at sample concentrations of 3 mg Chl/ml. The proton NMR-relaxivity measurements monitor the total proton population of the sample. However, since the water protons constitute the predominating species in the sample, the measurements basically reflect the relaxivity of the bulk phase water protons.

The manganese content of the samples was determined by neutron activation analysis as described in Ref. 18.

Reagent grade hexadecyltrimethylammonium bromide was obtained from Sigma Chemical Company.

Results

Reversal of the EDTA effect on the proton relaxivity

It is generally agreed that upon the addition of EDTA the enhancement in the proton relaxivity induced by photosynthetic membrane samples is greatly suppressed [9,16]. However, in our preparations the loss in the proton relaxivity by EDTA can be restored by simple washing of the samples with buffer medium in the absence of EDTA. Table I shows the $T_{1(corr)}^{-1}$/s and $T_{2(corr)}^{-1}$/s as well as the Mn content for pea thylakoid samples after isolation in the absence and presence of 2 mM EDTA during homogenization and after a number of washes with the EDTA-free standard high-osmotic buffer medium (the samples were not subjected to a low-osmotic wash in this experiment). A sample concentration of 200 μg Chl/ml was used during the washing procedure for these measurements. For the control samples, the $T_{1(corr)}^{-1}$/s, $T_{2(corr)}^{-1}$/s and Mn content decrease with each wash, as might be expected. However, for the samples isolated in the presence of EDTA, the $T_{1(corr)}^{-1}$/s and $T_{2(corr)}^{-1}$/s increase, while the Mn content decreases with the number of washes. After the third wash the values of the $T_{1(corr)}^{-1}$/s and $T_{2(corr)}^{-1}$/s approach what is observed for the control samples. These results show how it is

TABLE I

PROTON RELAXIVITY AT 27 MHz AND MANGANESE DETERMINATIONS OF CONTROL AND 2 mM EDTA TREATED PEA THYLAKOID SAMPLES SUBJECTED TO A SERIES OF WASHES WITH AN EDTA FREE, HIGH-OSMOTIC BUFFER MEDIUM

Wash number	Control samples washed at 200 μg Chl/ml			EDTA-treated samples washed at 200 μg Chl/ml			EDTA-treated samples washed at 1000 μg Chl/ml
	$T_{1(corr)}^{-1}$/s	$T_{2(corr)}^{-1}$/s	[Mn] (μg/mg Chl)	$T_{1(corr)}^{-1}$/s	$T_{2(corr)}^{-1}$/s	[Mn] (μg/mg Chl)	$T_{1(corr)}^{-1}$/s
0	0.976	3.772	–	0.244	2.392	–	0.240
1	0.628±0.132	3.476±0.054	0.817	0.287±0.050	2.455±0.138	0.780	0.242
2	0.552±0.068	3.127±0.042	0.628	0.616±0.066	2.664±0.192	0.755	0.284
3	0.490±0.050	3.096±0.022	0.507	0.539±0.039	3.033±0.196	0.648	0.306

possible to obtain a non-correlation between the proton relaxivity and Mn content of the samples upon treatment with EDTA. The interesting feature in the Mn data is that the presence of EDTA did not remove more Mn from our preparations than did simple washing with the standard buffer medium.

It was reported [9] that the reversibility of the EDTA effect could not be reproduced by simple washing. However, this appears to be due to different sample treament, since the chlorophyll concentration during the washing procedure is critical in order to observe the above effects. For comparison, Table I also shows the $T_{1(corr)}^{-1}$/s for a sample treated with 2 mM EDTA and then washed in the standard buffer medium at a sample concentration of 1 mg Chl/ml. After the third wash the $T_{1(corr)}^{-1}$/s still remains considerably suppressed with respect to the control value. Parts of Table I have been reported earlier [24].

Removal of extraneous, non-functionally bound manganese

The above considerations indicate that the sample preparation is relevant for the kind of effects that are observed in the proton relaxivity. A major difference in the sample preparation employed in Refs. 9–14 and Refs. 15–21 is the use of a wash in a low-osmotic buffer medium by the latter group. Table II gives the $T_{1(corr)}^{-1}$/s for seven different sample preparations before and after the samples were subjected to a wash in a low-osmotic buffer medium. The $T_{1(corr)}^{-1}$/s show a large variation after isolation among the different samples, in agreement with earlier work [10]. But after the wash in the low-osmotic buffer medium, the $T_{1(corr)}^{-1}$/s becomes rather consistent, as does the Mn content of the samples. The average Mn content in this case is similar to what was observed for samples subjected to three washes in the standard high-osmotic buffer medium (Table I). It is important to note that although the Mn content is similar in the above two cases, the $T_{1(corr)}^{-1}$/s is substancially higher in the samples subjected to a single wash with the low-osmotic buffer medium. The significance of this observation will be discussed later.

Table II also reports the O_2 activity for the samples before and after the low-osmotic wash. There is a loss of approx. 27% of the activity after the low-osmotic wash and this is reflected in the somewhat lower average Mn content of the samples, which corresponds to 3.24 Mn atoms per 400 Chl molecules. The commonly defined stochiometry for the functional Mn is about four Mn atoms per 400 Chl molecules [25]. However, similarly treated samples show only a small hyperfine Mn(II) EPR signal at room temperature (less than 10% of the Mn(II) EPR signal induced by NH_2OH treatment – e.g., see Ref. 21). Thus, damage to the functional Mn by the low-osmotic wash is minimal. Nevertheless, it is apparent from Table II that the extraneous Mn has been removed and, unless otherwise noted, thylakoid samples given one wash in a low-osmotic buffer medium were used in the

TABLE II

PROTON SPIN–LATTICE (T_1) RELAXATION AT 27 MHz, O_2 ACTIVITY AND MANGANESE DETERMINATIONS FOR SEVERAL PEA THYLAKOID PREPARATIONS SUBJECTED TO ONE WASH WITH A LOW-OSMOTIC BUFFER MEDIUM

Preparation number	Before Low-Osmotic Wash		After Low-Osmotic Wash			
	$T_{1(corr)}^{-1}$/s	O_2 activity (μmol O_2 per mg Chl per h)	$T_{1(corr)}^{-1}$/s	O_2 activity (μmol O_2 per mg Chl per h)	[Mn] (μg/mg Chl)	Mn atoms per 400 Chl
1	0.86	305	0.66	206	0.400	2.87
2	1.02	333	0.75	230	0.425	3.05
3	1.03	339	0.80	245	0.440	3.16
4	1.22	350	0.80	250	0.440	3.16
5	1.52	355	0.82	260	0.466	3.35
6	1.58	377	0.83	265	0.490	3.52
7	1.96	389	0.84	262	0.496	3.56
Average	1.31 ± 0.39	350 ± 28 [a]	0.78 ± 0.07	245 ± 21	0.451 ± 0.035	3.24 ± 0.25

[a] The O_2 activity in the absence of the uncoupler was 87 ± 12 μmol O_2/mg Chl per h.

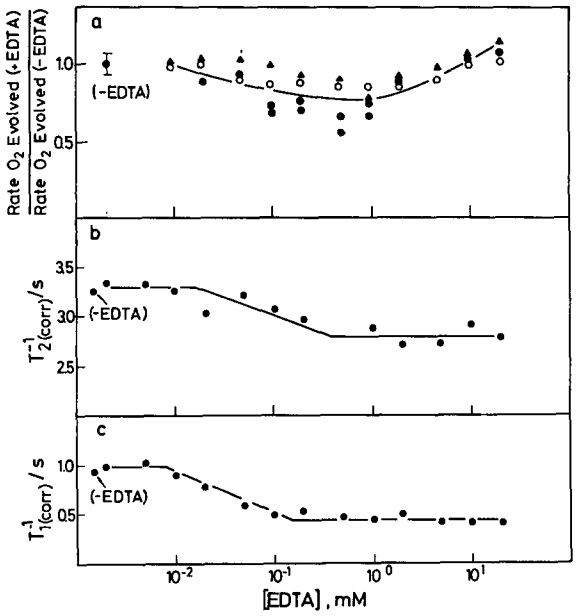

Fig. 1. Dependence of the proton relaxivity at 27 MHz and O_2 activity on EDTA concentration in thylakoid samples washed with a low-osmotic buffer medium. (a) O_2 activity; (b) spin–spin (T_2) relaxation; (c) spin–lattice (T_1) relaxation.

following experiments to study the effects of EDTA.

Dependence of the proton relaxivity on EDTA concentration

Fig. 1 shows the EDTA concentration dependence of the proton relaxivity as well as the O_2 activity for thylakoid samples. In Fig. 1b and c, for both $T^{-1}_{2(corr)}$/s and $T^{-1}_{1(corr)}$/s, respectively, there is a decrease with increasing EDTA concentrations in the range of 10–500 μM. Above 500 μM there is no further effect of EDTA on the proton relaxivity up to 20 mM concentration. The half maximal concentration for the EDTA effect on $T^{-1}_{1(corr)}$/s is approx. 30 μM and on $T^{-1}_{2(corr)}$/s it is approx. 100 μM. For our samples at 3 mg Chl/ml used in the NMR measurements, the Mn content corresponds to approx. 25 μM (see Table II). Thus, 4–20-fold more EDTA than the total Mn content of the sample is needed to cause the maximum suppression of the proton relaxivity enhancement. Typically, 1 mM or more EDTA is used in the treatment of photosynthetic membrane samples.

The interesting feature in Fig. 1b and c is that the extent of the loss in the proton relaxivity induced by EDTA is the same for both the T_1 and T_2 relaxation (corresponding to a maximum rate loss of approx. 0.5/s in each case), despite the fact that the absolute rate for T_2 relaxation is about 3-times greater than for the T_1 relaxation. This is expected from relaxation theory, since all relaxation mechanisms that lead to T_1 relaxation also contribute to T_2 relaxation and are additive [26,27]. This result indicates the T_2 measurements are as appropriate as T_1 measurements to monitor photosynthetic membrane samples, in contrast to earlier statements [14].

Fig. 1a shows the dependence of the O_2 activity of our samples on EDTA concentration. The O_2 activity exhibits a complex behavior in response to EDTA, showing a small decrease in the range of 10–100 μM and then a small increase in the range of 5–20 mM. The increase is not due to an uncoupling effect, since an uncoupler was included in the assay medium (see the Experimental Section). Within the scatter of the data points at 1–2 mM EDTA there is approx. 25% loss in the O_2 activity compared with the untreated control. However, as expected, there is no major inhibition of the O_2 activity in thylakoid samples by EDTA. Thus, upon EDTA treatment the correlation between the proton relaxivity and the O_2 activity appears to break down.

Kinetics of the EDTA effect on proton relaxivity

Fig. 2 shows the time dependence for the EDTA-induced decrease in the $T^{-1}_{2(corr)}$/s of thylakoid samples. As shown in Fig. 2a, the decrease in the proton relaxivity evolves only slowly with time upon the addition of 2 mM EDTA. The half-time is about 4.5 min in this sample. In Fig. 2b, 50 μM $MnCl_2$ was added to the sample to mimic the effects of extraneous, nonfunctionally bound Mn ions. The $T^{-1}_{2(corr)}$/s more than doubles in this situation, as expected [9,10]. Upon the addition of 2 mM EDTA, within the first 3 min, the $T^{-1}_{2(corr)}$/s drops to the original value observed before the addition of the $MnCl_2$, due to the rapid chelation of the added Mn. However, the $T^{-1}_{2(corr)}$/s continues to decrease with time, until it reaches a value similar to what was obtained for the sample in Fig. 2a. This further decrease has a half-time of about 7.5 min. From these significant kinetic dif-

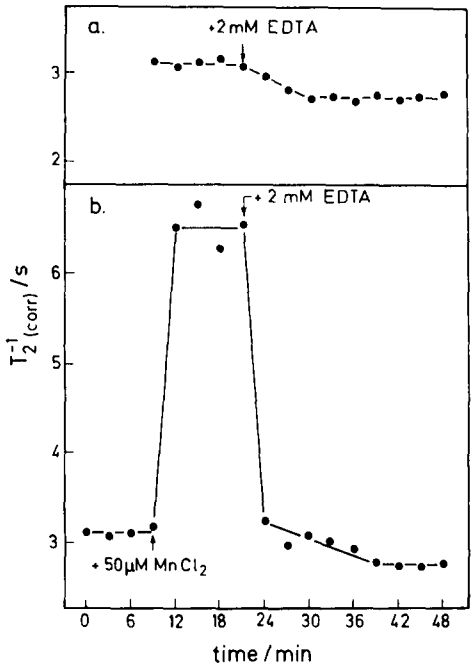

Fig. 2. Time dependence of the spin–spin (T_2) relaxation at 27 MHz upon the addition of 2 mM EDTA in thylakoid samples washed with a low-osmotic buffer medium. (a) Control; (b) after the addition of 50 μM $MnCl_2$.

ferences, we conclude that EDTA has additional effects on the proton relaxivity of thylakoid samples beyond the simple chelation of extraneous, non-functionally bound Mn ions.

Temperature dependence of the proton relaxivity

Fig. 3 shows the temperature dependence of the $T_{1(corr)}^{-1}$/s and $T_{2(corr)}^{-1}$/s at two different low-field proton resonance frequencies (16 and 27 MHz) for control thylakoid samples, samples containing 2 mM EDTA and samples subjected to a Tris-acetone wash. Tris-acetone washing is a severe treatment, and we do not know the full consequences of this treatment on thylakoid membranes. However, Tris-acetone washing does not solubilize much of the chlorophyll, but does extract about 2/3 of the Mn content of the sample [18] (as opposed to simple Tris washing which releases functional Mn from its native site, but does not remove it from the membrane [5]). As reported earlier, the proton relaxivity of Tris-acetone samples do not show a field dependence [18] and, as shown in Fig. 3 do not show a significant temperature dependence at the two

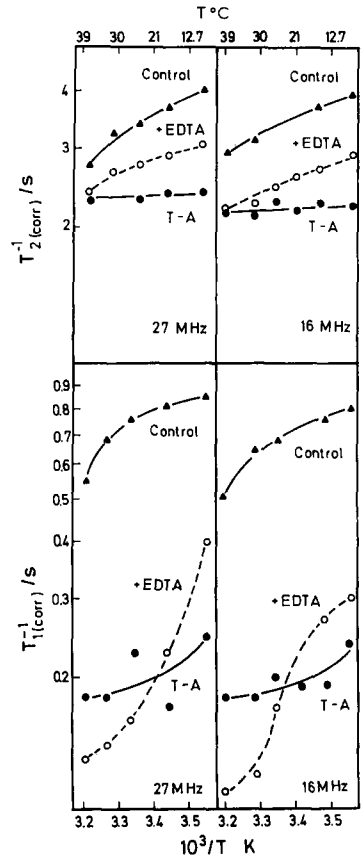

Fig. 3. Temperature dependence of the proton relaxivity at 16 and 27 MHz in thylakoid samples washed with a low-osmotic buffer medium. Measurements were made of controls, samples containing 2 mM EDTA, and samples subjected to a Tris-acetone (T-A) wash (see the Experimental section for details.).

proton resonance frequencies employed. Thus, Tris-acetone samples provide a good reference from which to estimate the contributions of the sample to the proton relaxivity that are not related to functional Mn. In terms of the manganese effect, this contribution will be referred to as the diamagnetic limit.

For control samples, there is not only a field dependence [18], but also a significant temperature dependence as well for both the $T_{1(corr)}^{-1}$/s and $T_{2(corr)}^{-1}$/s as shown in Fig. 3. The proton relaxivity increases as the temperature is decreased. This temperature behavior would indicate that the proton relaxivity is not slow-exchange limited. However, the proton relaxivity may lie within the intermediate-exchange region rather than the fast-exchange region (e.g., see Ref. 25). This situation

would mean that small variations in the spin-exchange rate could produce the slow-exchange limit, and hence reduce or mask any paramagnetic contributions to the proton relaxivity.

For the samples containing 2 mM EDTA, although the absolute values are considerably suppressed, the proton relaxivity still shows a measurable temperature dependence. These results would indicate that the proton relaxivity in this case does not reach the diamagnetic limit of the Tris-acetone samples, as assumed in Ref. 10.

Antagonistic interaction between the effects of EDTA and tetraphenylboron on the proton relaxivity

A number of chemical and physical treatments have been shown to increase the proton relaxivity of thylakoid samples including tetraphenylboron (TPB^-) [21]. Table III shows that the TPB^--induced enhancement in $T_{1(corr)}^{-1}$/s can be counteracted by treatment with EDTA. When samples are isolated in the presence of 2 mM EDTA, the enhancement is considerably suppressed upon the addition of TPB^-. But as the samples are carried through the wash procedure using the high-osmotic buffer medium as employed in the experiments in Table I, the increase in $T_{1(corr)}^{-1}$/s is restored upon the addition of TPB^-. After the second and third wash $T_{1(corr)}^{-1}$/s is comparable between the control and EDTA-treated samples, with or without TPB^-. Upon further addition of 2 mM EDTA to the samples, all enhancement in the $T_{1(corr)}^{-1}$, with or without TPB^- is suppressed. The recovery of the TPB^--induced enhancement in the proton relaxivity after washing EDTA-treated samples provides additional evidence that the proton relaxivity does not simply monitor phenomena exerted by extraneously bound Mn ions. The TPB^- induced enhancement was not observed in a previous study [10] due to the presence of EDTA in the samples. The origin of the TPB^- effect in the dark, however, remains to be clarified, since it was found that TPB^- affects only the higher S_2 and S_3 states of the water-oxidizing enzyme at low concentrations [28].

Proton relaxivity at 90 MHz of Photosystem II-enriched samples

According to relaxation theory [26,27], the paramagnetic interactions of bound Mn(II) to proton relaxation will correlate maximally at proton resonance frequencies under low-field conditions. This was shown to be the case for thylakoid samples not treated with EDTA from the frequency dispersion profiles [9,10,18], where a peak in T_1 relaxation was found to occur in the frequency range of 20–30 MHz. In the presence of EDTA there was no obvious corresponding peak in the low field frequency dispersion profile [9,10].

Based on relaxation theory we would not expect a significant paramagnetic contribution to the proton relaxivity at very high proton-resonance frequencies. However, we have measured the proton relaxivity at an intermediate frequency of 90 MHz (a magnetic field of 2.09 T). Table IV shows these results using a Triton X-100-prepared PS II-enriched sample. There are several points to

TABLE III

EFFECTS OF EDTA ON THE TETRAPHENYLBORON-INDUCED ENHANCEMENT OF THE PROTON SPIN-LATTICE (T_1) RELAXATION AT 27 MHz FOR PEA THYALKOIDS SUBJECTED TO A SERIES OF WASHES WITH AN EDTA-FREE, HIGH-OSMOTIC BUFFER MEDIUM

Wash number	$T_{1(corr)}^{-1}$/s			
	Control samples washed at 200 μg Chl/ml		2 mM EDTA-treated samples washed at 200 μg Chl/ml	
	$-TPB^-$	+5 mM TPB^-	$-TPB^-$	+5 mM TPB^-
0	0.890	1.608	0.368	0.651
1	0.788	1.221	0.327	0.640
2	0.627	1.280	0.692	1.164
3	0.531	1.372	0.502	1.386
+1 mM EDTA	0.290	0.290	0.166	0.211

TABLE IV

PROTON SPIN-LATTICE (T_1) RELAXATION AT 90 MHz OF SPINACH PHOTOSYSTEM II PREPARATIONS UNDER VARIOUS EXPERIMENTAL CONDITIONS

Sample	$T_{1(corr)}^{-1}$/s
Control	0.606 ± 0.022
Pellet	0.609 ± 0.024
Supernatant	0.075 ± 0.010
+1 mM EDTA	0.174 ± 0.054
+1 mM TPB^-	0.862 ± 0.018
+5 mM HTA^+	0.903 ± 0.033
Pellet	0.540 ± 0.020
Supernatent	0.309 ± 0.018

be considered from these data. First, the response of the $T_{1(corr)}^{-1}$/s to EDTA and to TPB^- follows the same behavior as observed for thylakoid samples at the low proton resonance frequencies (Table III). Second, the PS II-enriched sample control $T_{1(corr)}^{-1}$/s is higher at 90 MHz than might be expected from the data on thylakoids [18]. But we note that our PS II samples show about a 50% enrichment in the Mn content as determined by the heat-induced Mn(II) hyperfine ESR signal compared with thylakoid samples at the same Chl concentration (data not shown). Correspondingly, the O_2 activity of the PS II sample was higher at 480 μmol O_2/mg Chl per h. Finally, Table IV introduces the effect of a different compound, hexadecyltrimethylammonium (HTA^+). HTA^+ is an analogue compound of lauroylcholine which has been shown to interact specifically with PS II [29]. Upon the addition of HTA^+ under the experimental conditions employed, a significant enhancement in $T_{1(corr)}^{-1}$/s occurs, similar to what is induced by TPB^-. By contrast, TPB^- is an anionic species, while HTA^+ is a cationic species. The interesting feature with HTA^+ is that it can cause the release of the enhancement in $T_{1(corr)}^{-1}$/s into the supernatant, as opposed to the control.

From these results we conclude that the behavior of the proton relaxivity of photosynthetic membrane samples is similar at both low and intermediate proton resonance frequencies.

Discussion

Relaxation theory shows that the paramagnetic contributions to the proton relaxivity depend not only upon the concentration of bound paramagnetic sites and number of protons within the coordination sphere, but also upon the spin-exchange mechanisms between the bound protons and the solvent phase protons [26,27]. Although the photosynthetic membrane is an extremely complex system, water exchange across the membrane is sufficiently rapid with respect to the NMR time frame so as not to limit the effects of internally bound paramagnetic sites [18,30]. However, current photosynthetic models now locate the O_2-evolving site and the functional Mn to an internal compartment within the PS II protein complex [31,32]. Thus, the proton-spin exchange in the photosynthetic membranes will also depend upon the accessibility of internally bound paramagnetic sites, as well as the other spin exchange parameters.

One effect of EDTA is certainly to chelate extraneous, surface bound ions. But in this communication the results indicate that EDTA also produces additional effects on the membrane which is expressed through the proton relaxivity. In thylakoid samples which have had the extraneous, non-functionally bound Mn ions removed, treatment with EDTA causes a further suppression in both T_1 and T_2 relaxation, the rate loss being about equal for both relaxation measurements (Fig. 1). This additional effect of EDTA on the proton relaxivity can be kinetically resolved from the chelation function of EDTA (Fig. 2) and is reversible by thorough washing of the sample (Table I). Furthermore, this effect of EDTA can also reduce or mask the effects of other treatments on the proton relaxivity, such as the TPB^--induced enhancement (Table III). The proton relaxivity of thylakoid samples in the presence of EDTA still shows a measurable temperature dependence, indicating that the diamagnetic limit in the absence of functional Mn has not been reached (Fig. 3). And the EDTA effect is observable at intermediate proton resonance frequencies (90 MHz) (Table IV). We interpret this effect of EDTA on the proton relaxivity of photosynthetic membranes to arise from microenvironment changes which restricts the accessibility of internally bound paramagnetic sites to the solvent phase, at least in terms of the spin-exchange parameters within the NMR time frame. This restricted accessibility

probably obscures paramagnetically induced enhancements.

The importance of accessibility of internally bound paramagnetic sites on the proton relaxivity has been implicated in earlier experiments [21]. The opposite of the EDTA effect is evidenced in this paper by enhancements in the proton relaxivity produced through diverse treatments such as low-osmotic buffer medium (Table II), tetraphenylboron (Table III) or hexadecyltrimethylammonium (Table IV), Refs. 14 and 33 had used chelex, a large ion-exchange resin bead not expected to penetrate the membrane, in place of EDTA to remove surface-bound Mn ions. Under this condition as well, the proton relaxivity was considerably suppressed as with EDTA. We have had no experience with the use of chelex, but may suggest that effects of cationic chelators in general on the proton relaxivity is through secondary conformational phenomona related to the removal of surface-bound ions. Further experiments would be required to clarify this point.

A number of papers have indicated a correlation between O_2-evolution activity, functionally bound Mn and the proton relaxivity of the sample [15–21], whereas upon treatment of the samples with EDTA this correlation appears to break down [9–14]. The disappearance of the correlation between O_2 evolution and proton relaxivity by EDTA treatment could be caused by different phenomona. In this respect it should be mentioned that environmental effects can be very important for signals obtained by magnetic resonance methods as has been shown for the glycerol effect on the multiline EPR signal which reflects the S_2 redox state of the water oxidizing enzyme (for a discussion, see Ref. 34) and for the protonation and/or herbicide effects on the EPR signal arising from the Fe^{2+}-Q_A complex [35]. Furthermore, O_2-rate measurements are limited kinetically by the acceptor-side reactions, so that smaller effects on the water-splitting side could be masked. The data reported in this communication only show that the treatment with EDTA is not an appropriate experimental condition in using proton relaxivity as a tool to study the nature of the functional Mn.

In conclusion, we have shown that EDTA exerts a complex effect on photosynthetic membrane samples as monitored by the proton relaxivity. Since EDTA is often routinely added to samples to eliminate interference of extraneous Mn ions, we feel that it becomes relevant to evaluate all possible effects of EDTA, or other chelators, which may influence the Mn measurements of photosynthetic membrane samples.

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