Biochimica et Biophysica Acta, 408 (1975) 349—354

BBA Report

BBA 41274

WATER PROTON RELAXATION AS A MONITOR OF MEMBRANE-BOUND MANGANESE IN SPINACH CHLOROPLASTS

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(Received October 1st, 1975)

Summary

First measurements of proton relaxation on chloroplast membranes are presented here. Experiments show that the water proton spin-lattice relaxation rate in chloroplast thylakoid membrane suspensions can be used to monitor membrane-bound manganese. The relaxation effect is reduced to 0.4 of its original value upon manganese extraction by washing with either alkaline Tris buffer or NH_2OH/EDTA solution. Large increases in the proton relaxation rate are measured in the presence of reductants such as tetraphenylboron and NH_2OH; oxidants such as potassium ferricyanide or 2,6-dichlorophenolindophenol lead to a decrease in this rate. These results suggest that manganese exists as a mixture of oxidation states in dark-adapted chloroplasts.

Even though a considerable amount of kinetic information is available on the oxygen evolving mechanism in green plants, the actual chemistry remains a mystery. In the elegant kinetic model proposed by Kok and coworkers (see Joliot and Kok [1]), some chemical intermediate is assumed to accumulate up to four oxidizing equivalents in the light before reacting with water to yield oxygen. Since manganese is known to be essential for oxygen evolution and can take on a number of stable oxidation states, it may be acting as the so-called charge accumulating intermediate [2—5]. However, there has been no evidence for changes in the oxidation state of bound manganese during photosynthesis.

In order to resolve the function of manganese, it is necessary to have some means to monitor manganese in its bound state. Nuclear magnetic relaxation, particularly as applied to water protons, has been used quite success-

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Abbreviation: Cl_2Ind, 2,6-dichlorophenolindophenol.

fully in the study of paramagnetic ions, such as manganese, bound to biological macromolecules [6,7]. Using this technique we have found a close correlation between changes in the spin-latteice relaxation rate of water protons in chloroplast thylakoid membrane suspensions and the known behavior of membrane-bound manganese.

The application of pulsed NMR to studies of bound paramagnetic ions has been reviewed by Mildvan and Cohn [7]. Protons of molecules, e.g. H_2O coordinated to manganese, experience the large magnetic dipole of the paramagnetic ion's electron spin. The resulting electron-nuclear dipole-dipole interaction may be modulated by several time dependent processes leading to efficient relaxation of the nuclear spin level populations. The rate of nuclear relaxation by this mechanism is [8]:

$$\frac{1}{T_{1,M}} = \frac{C}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right) \tag{1}$$

where $T_{1,M}$ is the spin lattice relaxation time for nuclei of ligands in the first coordination sphere, r is the Mn-proton distance, ω_S and ω_I are the electron and nuclear Larmor frequencies respectively, C is a constant containing parameters of the magnetic dipolar interaction energy and τ_c is the correlation time for that interaction. τ_c itself has several components:

$$(1/\tau_c) = (1/\tau_R) + (1/\tau_S) + (1/\tau_M) \tag{2}$$

where τ_R is the rotational correlation time, τ_S is the electron spin relaxation time and τ_M is average lifetime of the complex; the shortest of these three characteristic times dominates.

Chloroplasts were isolated from market spinach (*Spinacea oleracea*) in 50 mM HEPES buffer, pH 7.5, 400 mM sucrose and 10 mM NaCl. They were given an osmotic shock and resuspended in the same medium. This provided a suspension of chloroplast thylakoid vesicles. All experiments were carried out on dark-adapted samples equilibrated to room temperature (23 ± 1°C). Measurements were made on 250 μl samples with a pulsed NMR spectrometer and probe (designed and constructed in Professor H.S. Gutowsky's laboratory), operating at 26.89 MHz. The inversion recovery method (180°-τ-90° sequence) for spin-lattice relaxation was employed [8], the 90° pulse width being 2.5 μs. The experimental uncertainty in the T_1 data is ≈ 5%.

Data for proton relaxation in the buffer medium and in a chloroplast suspension are shown in Fig. 1. $[M_o - M_z(\tau)]/2M_o$ is plotted vs. τ, the time after the 180° rf pulse, where $M_z(\tau)$ is the normalized amplitude of the free induction decay following the 90° pulse and M_o is the limiting amplitude ($\tau >> T_1$). The slope of the plot equals $-1/T_1$.

From Fig. 1 it is evident that the presence of chloroplast thylakoid membranes increases significantly the rate of proton relaxation over the buffer control. Since we are using chloroplast thylakoid vesicles, we may have observed two exponentials representing the inner and the outer compartments of the membrane vesicles. However, a single exponential is observed in all cases indicating that the bulk of water protons exchange sufficiently

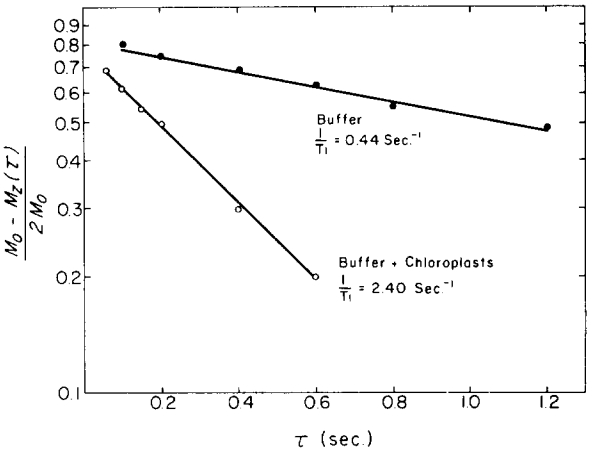

Fig. 1. Normalized height of the free induction decay signal, $(M_0-M_z)/2M_0$, plotted against τ, the time after the 180° rf pulse, for buffer and a chloroplast suspension. Chlorophyll concentration of chloroplast suspension was 3.0 mg/ml.

rapidly between different environments to yield a relaxation time which is the weighted average for water free in solution and water bound near paramagnetic sites in the membrane, i.e:

$$(1/T_1)_{obs} = \sum_i (1/T_{1,p})_i + (1/T_1)_{free} \qquad (3)$$

where $(1/T_1)_{obs}$ is the observed relaxation rate of the chloroplast suspension, $(1/T_{1,p})_i$ is the paramagnetic relaxation contribution for a given species i, and $(1/T_1)_{free}$ is the relaxation rate of uncomplexed H_2O.

The paramagnetic contribution for the i^{th} class of sites is given by Eqn. 4:

$$(1/T_{1,p})_i = \frac{[M_i]}{55.5}\left[\frac{q_i}{(T_{1,M})_i+(\tau_M)_i}\right] \qquad (4)$$

where [M] is the concentration of paramagnetic ion and q is the coordination number.

To facilitate comparisons all data in the tables and in the following discussion have been corrected for the relaxation rate in buffer alone or buffer plus the various reagents (values of $1/T_1$ for these solutions ranged from 0.40 to 0.44 s^{-1}). The reported relaxation rates, $1/T_1 = (1/T_1)_{obs} - (1/T_1)_{free}$, then, represent only the sum of paramagnetic relaxation contributions due to the chloroplast membranes.

Table I shows the $1/T_1$ values for several chloroplast preparations (from spinach leaves of different physiological age) having different Hill activities in saturating light. $1/T_1$ decreases approximately linearly with decreasing chloroplast activity but does not extrapolate through zero. Thus, the proton relaxation is monitoring a physiologically significant part of the chloroplast membranes as well as some residual component.

To determine the contribution of membrane-bound manganese to the observed relaxation, rate we measured $1/T_1$ on chloroplasts extracted of the

TABLE I

CORRELATION BETWEEN WATER PROTON SPIN LATTICE RELAXATION AND CHLOROPLAST O_2 EVOLUTION ACTIVITY

Sample No.	Hill Activity (μmol Cl_2Ind reduced/mg Chl/h)	Normalized to Sample 1	$1/T_1(s^{-1})$	Normalized to Sample 1
1	102	1.00	2.16	1.00
2	42	0.41	1.11	0.51
3	27	0.26	1.03	0.48
4	21	0.21	0.86	0.40

ion. Manganese extraction was accomplished by both Tris washing (0.8 M, pH 8) and NH_2OH/EDTA washing according to the procedures of Yamashita and Butler [9] and Ort and Izawa [10], respectively. The release of manganese by Tris washing has been demonstrated by the appearance of the $Mn(HOH)_6^{2+}$ ESR signal in the aqueous phase [11,12]. Although oxygen evolution is inhibited by these treatments, the rest of the electron transport chain remains intact and functional to the extent that variable fluorescence and photoreduction of $NADP^+$ or methyl viologen can be restored by exogenous donors [11,13]. The results given in Table II show that after manganese release, $1/T_1$ for the chloroplast membranes is reduced to about 40% of the control chloroplasts. Thus, it does appear that bound manganese makes a major contribution to the proton relaxation rate. However, we cannot, as yet, completely rule out contributions from other paramagnetic centers in the membrane, although we do not expect them to have a dominant effect. The paramagnetic effect on proton relaxation is limited by the accessibility to water. The copper in plastocyanin, for example, is not accessible to the bulk water [14] and should therefore make no contribution to the relaxation. With respect to iron, high spin Fe(III) is known to be much less efficient in relaxation in comparison with Mn(II) [6] so that the manganese effect is expected to predominate.

It is interesting to point out that the Tris-washing and NH_2OH/EDTA treatment(s) are known to remove only 60% of the bound manganese [2,12]. The remaining 40% effect on the $1/T_1$ may thus be due in part to the tightly bound fraction of manganese which is not removed. Another part of the residual effect could be due to the other paramagnetic centers in the membranes with a possible small contribution from the membrane-released $Mn(HOH)_6^{2+}$.

Several redox reagents have been shown to have a direct effect on the oxidation states of the charge accumulator for oxygen evolution in chloroplasts. Potassium ferricyanide has been used to oxidize all of the System II centers to Kok's S_1 state in dark-adapted chloroplasts [15]. The tetraphenylboron ion is known to directly reduce the positive holes on the charge accumulator, acting in competition with water as electron donor, but otherwise leaving the oxygen evolving apparatus and electron transport chain intact [16,17]. Similarly, NH_2OH is known to be photo-oxidized, donating electrons at or very near the System II reaction center [18]. We, therefore, looked at the effect of these redox reagents on the proton relaxation rate.

Table II lists the values of $1/T_1$ upon addition of oxidants and reductants to the chloroplast suspensions. With oxidants FeCy or Cl_2Ind the $1/T_1$ decreases.

TABLE II

THE EFFECTS OF MANGANESE EXTRACTION AND VARIOUS REDOX REAGENTS ON THE WATER PROTON SPIN-LATTICE RELAXATION RATE

Conditions	Chloroplast concentration (mg Chl/ml)	$1/T_1(s^{-1})$[a]	$1/T_1$(control)[b] (s^{-1})	Ratio $\frac{1/T_1}{1/T_1(\text{control})}$
Tris-washed[c]	1.0	0.13	0.32	0.41
	3.0	0.41	1.03	0.40
NH_2OH-EDTA washed[d]	1.0	0.08	0.18	0.44
Cl_2Ind, 0.3 mM	3.0	0.61	1.03	0.59
Potassium ferricyanide, 0.3 mM	3.0	0.57	1.03	0.55
NH_2OH, 0.2 mM	3.0	1.74	0.85	2.05
Tetraphenylboron, 0.5 mM	3.0	1.54	0.86	1.79
5 mM	3.0	2.16	0.86	2.51
Tris[c] + 0.2 mM NH_2OH	3.0	0.26	0.85	0.30
Tris[c] + 5 mM tetraphenylboron	3.0	0.31	0.85	0.36

[a] Water proton relaxation rate for the chloroplast suspension after treatment or with added reagents minus relaxation rate of the buffer alone or with reagent.
[b] Relaxation rate of chloroplast suspension in the absence of treatment. Variations in $1/T_1$(control) for the same chloroplast concentration are a function of Hill activity (see Table I).
[c] Chloroplasts washed in 0.8 M Tris pH 8, and resuspended in HEPES buffer, pH 7.5.
[d] Chloroplasts washed with solution of 50 mM HEPES, pH 7.5, 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA, 3 mM NH_2OH, and resuspended in HEPES buffer, pH 7.5.

On the other hand with the reductants tetraphenylboron or NH_2OH there is a very dramatic increase in $1/T_1$ over the control. The oxidants and reductants are probably affecting the manganese since there is no effect of the reductants of $1/T_1$ of Tris-washed chloroplasts (Table II). These results can be understood on the basis of the very different electron spin relaxation times, τ_S, of manganese in different oxidation states. For example for bound Mn(II),values of τ_S are generally 10^{-8}—10^{-9} s, the exact value being dependent on the NMR frequency and the chemical environment [6,7]. Mn(III), on the other hand, has a much shorter electron spin relaxation time. A recent study by Villafranca et al. [19] yielded a value of $\tau_S = 3 \cdot 10^{-11}$ sec for Mn(III) bound to a superoxide disumtase from *E. coli*. This difference in τ_S for Mn(II) and Mn(III) is sufficient to account for a much greater proton relaxation effect with the ion of lower oxidation state (Eqn. 1). Alternatively, differences in τ_M might procedure the observed effects (Eqn. 4). Moreover, we cannot yet ignore the possibility that the redox reagents may exert secondary, structural effects on the paramagnetic centers such that water accessibility is altered.

Taking all the arguments into consideration, the results suggest that there may be a mixture of manganese oxidation states in the dark-adapted chloroplasts. The most common oxidation states of manganese are Mn(II), Mn(III) and Mn(VII) [20], while Mn(II) and Mn(III) are probably the most significant biologically. Both Mn(II) and Mn(III) form stable complexes, but these oxidation states can be easily interchanged under the appropriate chemical environment [20]. The charge accumulator for oxygen evolution may be a multiple

manganese complex, as suggested by Earley [5], having both Mn(II) and Mn(III). If this is the case, then a decrease in the relaxation rate would be expected upon the addition of an oxidant which oxidizes the fraction of Mn(II) to Mn(III). However, an increase in the relaxation rate is expected upon the addition of a reductant which reduces the fraction of Mn(III) to Mn(II). Thus, there seems to be a good correlation between the predicted action of these redox reagents on manganese oxidation states as monitored by the proton relaxation and their known effects on the charge accumulator.

In this paper we have introduced NMR relaxation as a useful tool in the study of bound manganese in chloroplast thylakoid membranes. We are currently undertaking an investigation of the proton relaxation after brief flashes of light in order to determine whether or not manganese can be directly related with the charge accumulator of oxygen evolution in photosynthesis.

We are grateful to Professor Gregorio Weber and H.S. Gutowsky for discussions and Professor Gutowsky for the use of his research facilities. We are thankful for financial support from the National Science Foundation (GB 36751) to G and (MPS 73-04984) to HSG, the National Institutes of Health (GM 18038) to PGS, and the Office of Naval Research (NR 056-547) to HSG. TW was partially supported by HEW PHS GM7283-1 (Sub. Proj. 604) training grant in Cellular and Molecular Biology.

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