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NMR RELAXIVITY CHANGES IN CHLOROPLAST SUSPENSIONS

EFFECTS OF NH_2OH AND OF TREATMENTS ALTERING THE REDOX STATE OF THE PHOTOSYNTHETIC ELECTRON TRANSPORT CHAIN

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Summary

Treatments (illumination, chemical oxidation or reduction) which are potentially capable of producing paramagnetic centers in chloroplast thylakoid membranes do not produce enhancements of the proton magnetic relaxivities of these preparations. However, exposure of thylakoid membranes to varying concentrations of hydroxylamine induces a time-dependent increase in relaxivity for which the steady-state magnitude is dependent on hydroxylamine concentration. The appearance of relaxivity is correlated kinetically with inactivation of oxygen-evolving centers; in addition both processes show a threshold effect with respect to hydroxylamine concentration. Kinetic analyses of these hydroxylamine-induced effects suggest that at low ($\leqslant$100 μM) and at intermediate (200—500 μM) concentrations, hydroxylamine extraction is partially counteracted by a reverse process that reactivates oxygen-evolving centers in the dark.

Introduction

Oxygen evolution by PS II is proposed to procede by a sequential four-step mechanism comprised of a set of so-called 'S-states', each of which (S_0, S_1, S_2, S_3, S_4) represents a higher oxidation state of the oxygen evolving system [1—4]. A number of experimental observations suggest that manganese is involved in the water-splitting reaction; these include inactivation of the reaction by Mn

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS, photosystem; Chl, chlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine.

extraction with Tris [5—13] or NH_2OH [5,12,14,15], impairment of oxygen evolving activity in whole plants [5,16] and algae [17—20] by Mn deficiencies, and restoration of oxygen evolution activity in depleted chloroplasts [16] and algae [19—21] by readdition of Mn. By virtue of its strong enhancement of the nuclear magnetic relaxation rate of solvent protons [22], Mn is ideally suited to studies, by the NMR technique, of phenomena associated with this ion and its possible role in photosynthetic oxygen evolution.

In a previous report [23], we have shown that the water crossing time of the thylakoid membrane is sufficiently rapid to permit measurements of relaxivity on the interior side of the thylakoid membrane, where the oxygen-evolving complex is generally believed to reside [24,25]. We have also shown that chloroplast thylakoid membranes isolated in the presence of EDTA retain high rates of oxygen evolution and photophosphorylation activity [26], but in contrast to other reports [27—35], we find that such chloroplast membrane preparations are devoid of the maximum in the region from 20 to 30 MHz of the dispersion profile (R_1 vs. Larmor frequency) which is characteristic of Mn(II) bound to sites of reorientationally restricted mobility. The results of our studies on chloroplast nuclear magnetic relaxivities to date [26,36] indicate that Mn functional in the water-splitting reaction of PS II does not influence proton magnetic relaxation rates in dark-adapted chloroplasts. This fact is consistent with any of the following interpretations: manganese in the water-splitting complex is present (a) in solvent-inaccessible sites: (b) as Mn(II) in solvent-accessible sites where exchange of protons between bulk solvent and the hydration sphere of manganese is slow on the NMR time-scale; (c) in a solvent-accessible form bound to a stable, higher oxidation state of low molar relaxivity, such as Mn(III).

In this communication we present data on the relaxivity of chloroplast suspensions after illumination, and after a variety of chemical treatments that alternately oxidize and reduce the chloroplast electron transport chain. These experiments show no measurable relaxation enhancements attributable to membrane-bound paramagnetic centers outside the water-splitting complex. Although Mn(II) associated with the oxygen evolution reaction is not monitored by NMR in untreated, dark-adapted chloroplasts, we have presented preliminary data [26] showing that exposure of thylakoid membranes to NH_2OH produces a large relaxivity enhancement, that this relaxivity is only partially suppressed by addition of EDTA, and that the dispersion profile of this signal is characteristic of bound Mn(II) [26]. Data are presented in this communication which show that the relaxivity induced by NH_2OH is closely correlated with Mn(II) associated with the oxygen evolving system of PS II.

Materials and Methods

Broken chloroplast thylakoid membranes were prepared by a modification of the method of Robinson and Yocum [37] in which 1 mM EDTA was included in the homogenization buffer. The isolated, washed membranes were suspended in 20 mM Tricine (pH 8) containing 15 mM NaCl and 400 mM sucrose, and stored at —70°C in 0.5 ml aliquots prior to use in the experiments described here. The effect of NH_2OH on thylakoid membrane relaxivities was

examined by adding aliquots of a 100 mM solution of hydroxylamine (in 0.01 N HCl), whose pH was adjusted to 7.5 just prior to use, to 200 μl of chloroplast suspensions in NMR tubes. Exposure of thylakoid membranes to NH_2OH and all ensuing NMR measurements were carried out at 25°C. For the measurement of dark inactivation of O_2 evolving centers by oxygen polarography, chloroplasts equivalent to 2.6 mg/ml chlorophyll were incubated in the dark at 25°C with NH_2OH (pH 8.0) for varying lengths of time. Aliquots (10 μl) of this suspension were transferred to an oxygen electrode cuvette, and incubated 15 s in the dark prior to exposure to saturating red illumination [35,37]. The reaction vessel (1.5 ml) contained: 66 mM NaCl, 53 mM Tricine (pH 8.0), 3.3 mM $MgCl_2$, 1.3 mM NaCN, 66 μM methylviologen, and 5 μg gramicidin D.

The apparatus for NMR relaxation measurements has been described previously [36]. Spin lattice relaxation rates, R_1, of solvent protons were measured at 20.7 MHz using the modified triplet sequence [38]. Data were digitized and analyzed by a least-squares fitting procedure using a minicomputer. The procedures for measurements of oxygen evolution activity and light-induced cytochrome *f* oxidation/reduction changes have been described previously [39]. Illumination of NMR samples utilized a beam of white light ($>10^6$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}) filtered through 2.5 cm of a 0.2% $CuSO_4$ solution. Where indicated, this beam was passed through a 720 nm Balzers interference filter. NMR measurements were at 25° C.

Results

Effect of oxidation-reduction changes on thylakoid membrane relaxivities

A number of components of the photosynthetic electron transport chain undergo one-electron oxidation-reduction reactions to generate paramagnetic species which could in principle influence solvent proton relaxivities. Species with paramagnetic spins of one-half, in particular, could provide a relatively efficient relaxation pathway for interacting nuclei due to the relatively long paramagnetic relaxation times typical of such species [40]. Spin-1/2 species present in thylakoid membranes include organic radicals (plastosemiquinone anions, chlorophyll radical cations) as well as Cu(II) in oxidized plastocyanin, or in ribulosebisphosphate carboxylase, if any of the latter were retained in our broken chloroplast preparations. The reduced iron-sulfur centers A and B associated with the primary acceptor function in PS I may also contain exchange-coupled Fe(III)-Fe(II) pairs in a formal spin-1/2 state [41]. To examine the possible influence of such species on proton relaxivity, we have alternately oxidized and reduced portions of the electron transport chain by illumination, or through the use of added chemical oxidants and reductants. Since the thylakoid membranes used for these experiments were isolated in the presence of EDTA, spurious signals from non-functional, loosely-bound Mn(II) [36] would not be observed. The lower portion of Fig. 1 compares proton relaxivities in thylakoid suspensions that were illuminated anaerobically with strong white light ($>10^6$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}) in the absence of an electron acceptor, with the relaxivity of the same suspension after aeration, addition of an electron acceptor (benzylviologen), and exposure of the suspension to monochromatic light (720 nm). Note that deoxygenation of the suspension with water-saturated

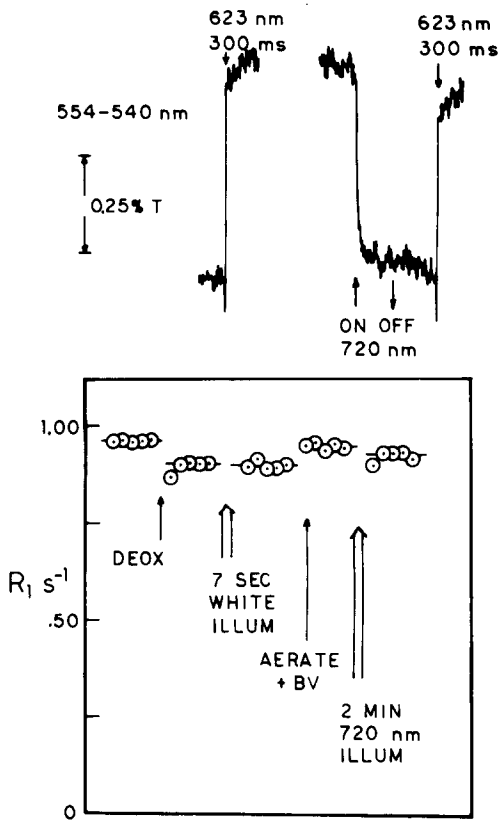

Fig. 1. Lower portion: Effects of various illumination protocols on the proton relaxation rate (R_1) of a chloroplast suspension (2.85 mg Chl/ml). Upper portion: Oxidation-reduction changes of cytochrome *f* observed on the same suspension under identical conditions of illumination. See text for experimental details.

nitrogen gas produces a small decrease in relaxivity (0.07 s^{-1} at 25°C) owing to a paramagnetic contribution from dissolved oxygen. Broad-band white light illumination (7 s) of the deaerated suspension, which should reduce carriers in the electron transport chain between PS II and PS I, produces no change in relaxivity. Subsequent aeration produces an increase in relaxivity from the introduction of oxygen to the sample, but no further changes are seen upon exposure to 720 nm light, which oxidizes the electron transport carriers situated between PS II and PS I.

The upper portion of Fig. 1 presents the results of an optical experiment which monitors cytochrome *f* changes in our thylakoid suspensions, and which serves as a control to demonstrate that the illumination protocol, with 720 nm light, of dense chloroplast suspensions required for the NMR experiment (2.85 mg Chl/ml) serves to oxidize the electron transport chain. The tracing on the upper left of Fig. 1 was obtained by (1) exposing the concentrated thylakoid suspension in an NMR tube (10 mm outer diameter) to 720 nm light for 2 min and (2) transferring an aliquot (90 μg Chl) from this tube (in the dark) to 1 ml of reaction mixture (20 mM Tricine, pH 8 containing 50 mM NaCl) in an Abel-

TABLE I

Effect of redox changes on proton relaxation rate (R_1) of chloroplast suspensions. Conditions of illumination are given in the legend of Fig. 1. The suspension of Experiment I contained 20 mM Hepes, rather than Tricine, as buffer.

		$R_1 \pm \sigma$	n
I	Chloroplasts (3.20 mg Chl/ml)	0.957 ± 0.011	4
	+ 1 mM EDTA	0.957 ± 0.003	4
	+ 50 μM DCMU	0.952 ± 0.016	4
	30 s white illumination	0.909 ± 0.006	4
II	Chloroplasts (2.85 mg Chl/ml)	0.918 ± 0.003	4
	after deoxygenation	0.850 ± 0.019	4
	+ 1 mM EDTA	0.790 ± 0.008	4
	+ 5 mM dithiothreitol	0.752 ± 0.006	4
	+ dithionite	0.743 ± 0.005	4
III	Chloroplasts (2.70 mg Chl/mg) deoxygenated		
	+ 0.5 mM EDTA	0.801 ± 0.007	4
	+ 5.0 mM $Fe(CN)_6^{-3}$	0.816 ± 0.012	4
IV	Chloroplasts (2.23 mg Chl/mg)		
	+ 5 mM EDTA	0.764 ± 0.013	2
	+ 0.5 mM $NaB(C_6H_5)_4$ (2.5% dilution)	0.746 ± 0.023	3
	+ 1.5 mM $NaB(C_6H_5)_4$ (10% dilution)	0.716 ± 0.004	3

son cuvette (1 cm pathlength) where it was subsequently exposed to a single 300 ms flash of 623 nm light. The right-hand tracing shows the result obtained with the same sample in the reaction mixture upon exposure first to 720 nm light (after the preceding 623 nm flash), which again oxidizes the cytochrome *f*, and the effect of a subsequent 623 nm flash, which is seen to reduce the oxidized cytochrome *f*. It is apparent from these control experiments that the method used here is sufficient to produce photooxidation of electron carriers between PS II and PS I, and that neither this procedure, with 720 nm light, nor anaerobic illumination with strong white light produces paramagnetic species that contribute significantly to proton relaxivities.

Similar experiments were carried out using a variety of procedures summarized in Table I. Experiment I shows the effect of white light illumination on DCMU-treated chloroplasts. Since DCMU blocks the photoreduction of the quinone pool by PS II and strongly retards reoxidation of the acceptor side of PS II after illumination [42,43], this experiment examines the effect of redox changes in the acceptor side of the PS II trap between the primary acceptor and the DCMU block. Illumination has only a very slight effect on R_1 (0.04 s^{-1}), and the observed change is a decrease, rather than an increase, in relaxivity. Since the reduced, rather than the oxidized form of the PS II acceptors are thought to be paramagnetic (as plastosemiquinone anions [44,45]), any change in R_1 associated with reduction of this portion of the electron transport chain should be an increase, rather than the observed decrease. The change is very small however, and probably results from partial deoxygenation of the suspension during illumination.

The effects of dithiothreitol ($E'_0 = -0.330$ V) and dithionite on thylakoid relaxivities are shown in experiment II (Table I). After small initial decreases

due to deoxygenation and EDTA addition (which chelates residual traces of free paramagnetic ions), dithiothreitol produces only a slight further reduction in R_1 of 0.04 s^{-1}. Since its midpoint potential is higher than that of the primary acceptor of PS I, or of the iron-sulfur centers A and B [46,48], dithiothreitol should leave the electron transport chain reduced with the exception of the acceptor side of PS I. Dithionite should reduce the acceptor side of PS I along with the rest of the photosynthetic electron transport chain. The fact that dithionite produces no change in R_1 with respect to the values for control chloroplasts when added to chloroplast suspensions after dithiothreitol (Experiment II), indicates that the reducing side of PS I does not contribute significantly to the observed relaxivity.

Two redox agents that have previously been reported [27,29—31] to cause substantial relaxivity enhancements (ferricyanide and tetraphenylborate) were also studied (experiments III and IV of Table I). Neither reagent at the concentrations shown produced a significant enhancement of relaxivity in chloroplasts and in the presence of EDTA. The slight decrease seen in the presence of tetraphenylborate is attributable entirely to dilution effects. The discrepancy between the present and previous results may be due either to differences in the preparative procedure used to obtain thylakoid membranes, especially with respect to the presence of EDTA in the isolation and resuspending buffers [26,36], or alternatively to the different concentrations of these redox agents employed.

Effects of hydroxylamine on thylakoid relaxivities

The results presented in the preceding section demonstrate that oxidation-reduction changes in the photosynthetic electron transport chain do not induce appreciable changes in the relaxivities of thylakoid suspensions. We next sought to characterize relaxivity changes produced by inactivation of the water-splitting reaction with NH_2OH. Previous investigations have shown that this reagent produces two distinct effects on the oxygen evolution reaction in isolated thylakoid membranes and in intact algae. At low concentrations ($\lesssim$50 μM), NH_2OH retards by two flashes the oscillations in the flash-dependent oxygen yield [49,50] but has little or no effect on the steady state rate of water oxidation [51]. This effect has been interpreted in terms of the reduction of the S-states to S_0 by a single bound NH_2OH with subsequent photooxidation of the bound species [50]. A very similar effect observed in the presence of H_2O_2 was interpreted to indicate that the oxidation pose of the S-states had been altered to a more highly reduced (S_{-1}) form [52]. At higher concentrations (1—10 mM), NH_2OH destroys oxygen evolution activity with concomitant release of two-thirds of the thylakoid-associated Mn(II) [5,12].

Figs. 2 and 3 illustrate the time-dependent effects, obtained upon exposure of thylakoid suspensions to varying concentrations of NH_2OH, on nuclear magnetic relaxivities. These experiments were conducted in the dark using chloroplast thylakoid suspensions isolated in the presence of 1 mM EDTA to remove non-functional paramagnetic ions [26,36]. Fig. 2 shows that low concentrations of NH_2OH (100—200 μM) produce little, if any, increase in R_1. At concentrations at or above 200 μM, on the other hand, time-dependent increases in R_1 are apparent; the extent of these increases as well as the time required to

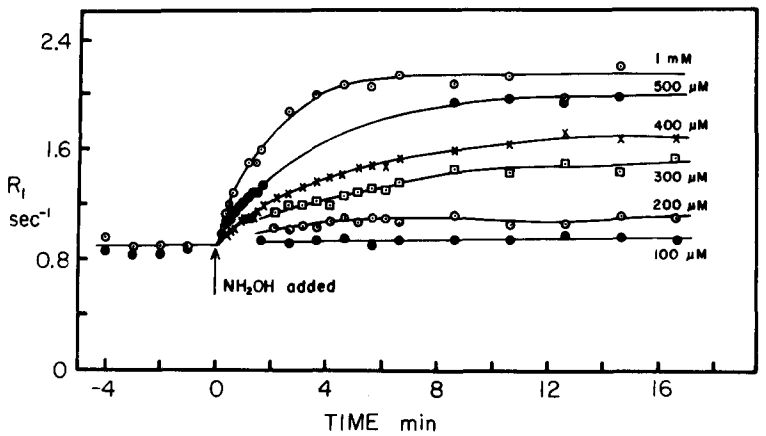

Fig. 2. Time course of the changes in the nuclear magnetic relaxation rate of solvent protons in chloroplast suspensions (2.3 mg Chl/ml) produced by addition of low and intermediate concentrations of NH_2OH. See Materials and Methods for the experimental procedure.

achieve maximal relaxivity are dependent on the concentration of NH_2OH present. In Fig. 3, the effects of high concentrations (1, 2, and 5 mM) of NH_2OH on proton relaxivity are shown. These concentrations have previously been shown [5,15,51] to inhibit oxygen evolution by extraction of Mn(II), and in the experiments shown induce a relatively rapid enhancement of R_1. The maximum enhancement, reached after a few minutes of incubation, is essentially independent of the concentrations of NH_2OH employed here.

The observation that a threshold concentration of NH_2OH is required for the appearance of enhanced relaxivity suggested that a similar effect might be present in the NH_2OH-induced destruction of oxygen evolution activity. Fig. 4 compares the proton relaxivity and oxygen evolution activity achieved after incubation of broken chloroplasts for 16 min in various concentrations of

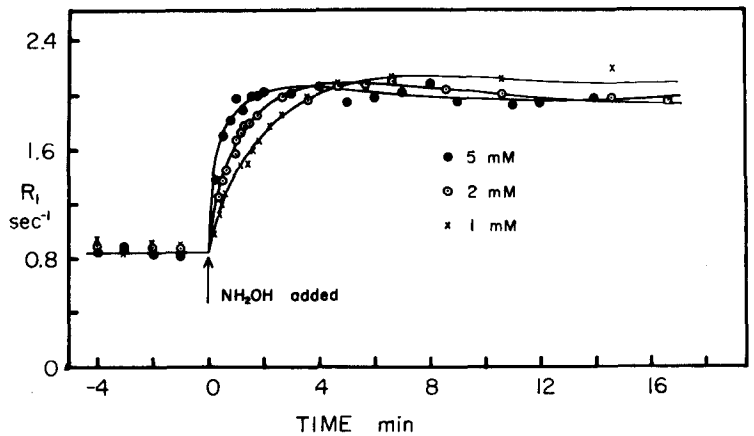

Fig. 3. Time course of the changes in the nuclear magnetic relaxation rates of chloroplast suspensions exposed to high concentrations of NH_2OH. Other conditions are described in the legend to Fig. 2.

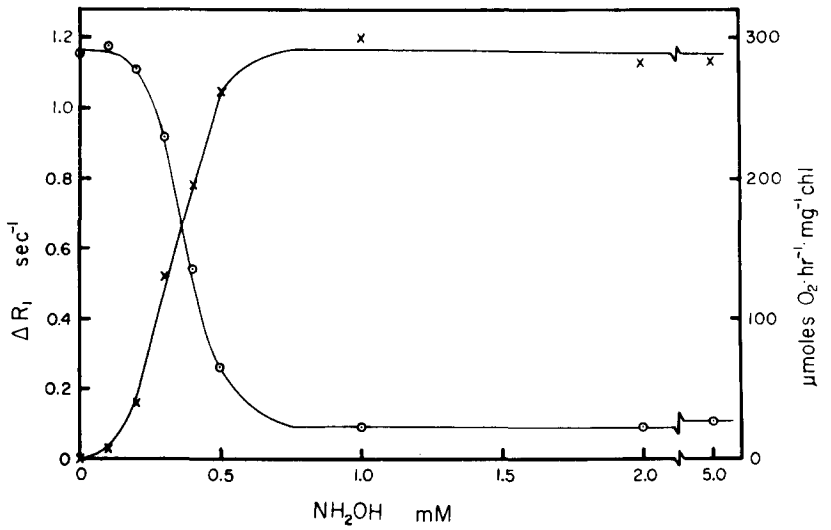

Fig. 4. Comparison of the effects of varying concentrations of NH_2OH on destruction of oxygen evolving activity (○) and the change in R_1 (X). The experiments were conducted on identical samples of chloroplasts (2.7 mg Chl/ml); exposure to 1.0 mM NH_2OH and subsequent measurements were carried out at 25°C; the values shown were measured after 16 min of incubation. ΔR_1 is $|R_1 - R_1$ (init)|, where R_1-(init) is the initial relaxation rate observed before incubation in NH_2OH.

NH_2OH. The close correlation between these experiments, including the threshold concentration near 100 μM and the fact that the maximal inactivation effect as well as the change in R_1 are achieved in both experiments at similar concentrations (between 0.5 and 1.0 mM), leaves little doubt that NH_2OH-induced proton magnetic relaxation measurements monitor events that are concomitant with the destruction of oxygen evolving centers and the appearance of solvent-exposed, membrane-bound Mn(II).

The process by which NH_2OH inactivates oxygen evolution activity does not appear to occur at concentrations less than 100 μM, even though NH_2OH is known to interact with the S-states at these concentrations [49—51]. At concentrations of NH_2OH below 500 μM, only a portion of the Mn is exposed as a relaxation-producing species, even at quite long times after addition of the reagent. Cheniae and Martin [15] have reported that the kinetics of the inhibition of oxygen evolution by NH_2OH are complex; in the presence of the reagent, inactivation of oxygen evolution shows a sudden initial decrease followed by apparent first-order kinetics. The rate of inhibition during the slow phase is first-order with respect to NH_2OH concentration. Relaxation enhancements following addition of NH_2OH to dark-adapted thylakoid membrane suspensions at 25°C exhibit a simple monophasic approach to the steady-state relaxivity (Fig. 5). Close inspection of data obtained on suspensions with somewhat higher chlorophyll concentrations indicates a small deviation from strictly exponential kinetics. These more subtle effects will be discussed elsewhere. A rapid phase in the kinetics is clearly absent from the data at 1, 2, and 5 mM NH_2OH, although the data obtained at lower concentrations (300—500 μM) could be consistent with a small (about 15%) rapid component. The rates of

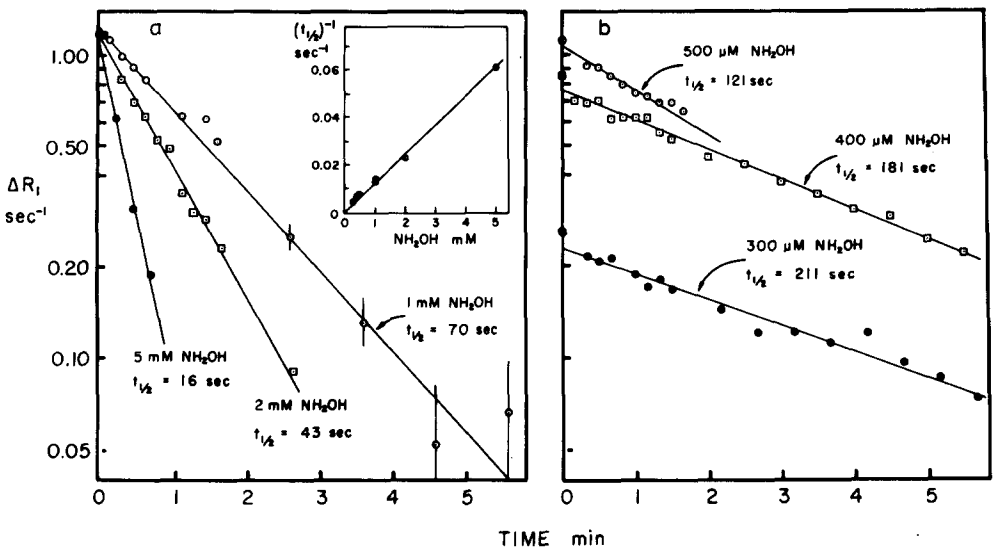

Fig. 5. Kinetics of the appearance of NH_2OH-induced relaxivity of chloroplasts (2.3 mg Chl/ml) at (a) high and (b) low to intermediate concentrations of NH_2OH. Inset: Inverse half-time for the appearance of enhanced relaxivity as a function of NH_2OH concentration.

change of R_1 are quantitatively very similar to those found for the slow phase of the destruction of oxygen evolving centers by Cheniae and Martin [15] at the same temperature; for example, when the NH_2OH concentration is 1 mM, the $t_{1/2}(R_1) \cong 70$ s and the $t_{1/2}$ for inactivation of oxygen evolution is 75 s [13]. Moreover, the rate constants for both processes are approximately first-order in NH_2OH (Fig. 5 (inset) and Ref. 13).

A kinetic study of the inhibition of oxygen evolution in the presence of 1 mM NH_2OH in the dark at 25°C is shown in Fig. 6. The kinetics are monophasic and do not show the rapid initial inhibition observed by Cheniae and Martin [13]. The rapid initial phase of inactivation observed by these workers

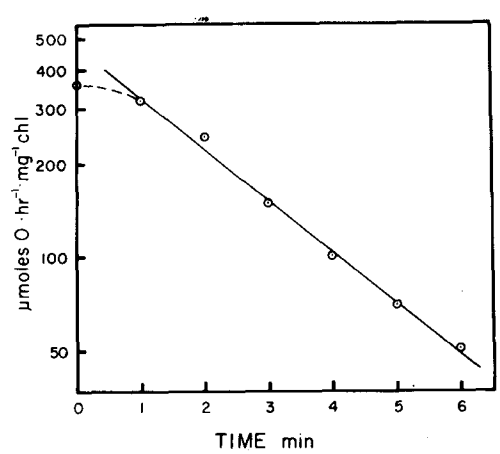

Fig. 6. Rate of inhibition of oxygen evolution activity by 1 mM NH_2OH.

was not present in our experiments; rather, there was a delay (<1 min) in the onset of NH_2OH action followed by apparent first order kinetics. The half-time for inactivation ($t_{1/2}$ = 105 s) is similar, but not identical to the rate constant for the appearance of proton relaxivity ($t_{1/2}$ = 70 s) and the slow phase ($t_{1/2}$ = 75 s) of the inhibition of O_2 activity observed by Cheniae and Martin. From the general similarity of the rates of these processes it seems very likely that both the NMR experiment and oxygen uptake measurements monitor the same reaction leading to destruction of the O_2-evolving centers, and that the differences result from the assay procedures in O_2 measurements. The measurements of Figs. 5 and 6 were obtained using a single preparation of chloroplasts under identical conditions of extraction except for the assay procedure for oxygen evolution activity. This procedure involved dilution of the chloroplasts into the assay mixture, followed by a 15-s dark delay and subsequent oxygen uptake measurements in the light. The change of supporting medium with concomitant dilution of the NH_2OH, the ensuing dark delay, and perhaps even the exposure to light inherent in the assay procedure may have lengthened the apparent kinetic of NH_2OH-induced inhibition of oxygen evolution activity. This possibility is discussed further below.

Discussion

The electron transport chain of chloroplast thylakoid membranes contains several redox carriers potentially capable of forming paramagnetic centers which, if solvent-exposed, could contribute to proton magnetic relaxation rates. This possibility has been tested (Fig. 1, Table I) by measurements of R_1 under conditions (light, oxidants, reductants) where such paramagnetic centers might be produced. We have examined (1) the reducing side of PS II up to the DCMU block; (2) components between PS II and PS I; and (3) the reducing side of PS I. Save for small changes in relaxivity which may be attributed to either the addition, or removal, of oxygen from our samples, or to chelation of traces of non-functional paramagnetic ions by EDTA, we can find no species in intact, functional chloroplasts which make significant contributions to the proton magnetic relaxivities of these preparations. Our failure to observe enhanced relaxivities with ferricyanide or tetraphenylborate are in contrast to other reports [27—30], in which relatively high concentrations of these reagents were employed and in which the potential effects of loosely-bound nonfunctional Mn(II) were not suppressed by EDTA.

In studies reported elsewhere [26,36] we have shown that chloroplasts prepared in the presence of 1 mM EDTA show no characteristic maxima in dispersion profiles of R_1. We have additionally shown that exposure of thylakoid membranes to Tris plus EDTA in the light, which inactivates oxygen evolution activity, does not appreciably alter the dispersion profiles obtained for native, oxygen-evolving membranes also capable of carrying out photophosphorylation. From results such as these it appears probable that Mn in the oxygen-evolving complex associated with PS II in dark-adapted chloroplasts does not contribute appreciably to proton relaxivity.

We have previously reported [26] that exposure of physiologically active suspensions of thylakoid membranes to NH_2OH elicits an enhancement of the

nuclear magnetic relaxation rate that is partially sensitive to EDTA, and that exhibits a dispersion profile characteristic of Mn(II) bound to sites of reorientationally restricted mobility. These observations are confirmed and substantially extended by the data shown in Figs. 2 and 3, which demonstrate both the time- and NH_2OH concentration-dependence of the appearance of enhanced relaxivity. The data in Fig. 4 show a close correlation between the increase in R_1 and destruction of O_2 evolution activity. From these results we conclude that the enhancement in R_1 reported here arises from Mn(II) which was functionally associated with the oxygen-evolving apparatus prior to NH_2OH treatment.

A surprising feature of our data (Fig. 2) is the apparent protection from NH_2OH inactivation of activity observed at low concentrations ($\leqslant 100$ μM) of the reagent, as well as the apparent partial protection that occurs at intermediate NH_2OH concentrations (200—500 μM). For example, at 300 μM NH_2OH, the first-order rate constant for inactivation obtained from the data in Fig. 5 implies that the inactivation process is about 86% complete after 15 min; the liberation of Mn(II), monitored by R_1, as well as the destruction of oxygen-evolving centers, measured as oxygen evolution, are however, only 30 to 40% of the maximal levels observed at high (1—5 mM) NH_2OH concentrations. It would appear, therefore, that the destructive effect of NH_2OH on activity and Mn(II) liberation is countered by some reverse process that is relatively effective at low NH_2OH concentrations. Since the extraction process is first order with respect to NH_2OH, the reverse process is clearly less than first-order with respect to this reagent.

It is significant that the observed threshold effect (Fig. 4) cannot be explained by an inhibition of the rate of NH_2OH extraction at low concentration, as might be expected from a cooperative mechanism. The rate of inactivation of oxygen-evolving centers at intermediate NH_2OH concentrations (300—500 μM) is sufficiently rapid to ensure attainment of steady-state conditions, but the increase in R_1 and the loss of oxygen-evolving capacity is substantially less than the maximal changes observed at high (>1 mM) NH_2OH concentrations. Thus, the threshold observed in Fig. 4 is not a cooperative inactivation process where added NH_2OH facilitates destruction of oxygen-evolving centers, but instead, appears to reflect a steady-state condition where rates of inactivation and restoration of activity are equal.

The reason for the initial lag observed in the onset of NH_2OH-induced inhibition of O_2 evolution activity (Fig. 6) and for the lengthened kinetic of inhibition with respect to the enhancement of R_1 (Fig. 5) is not clear at present. As pointed out above, the assay procedure for O_2 evolution may be involved, particularly if the dark repair process inferred from the kinetic data of Figs. 2—4 occurs in the assay medium after dilution of NH_2OH. An active repair process, unopposed by the extraction reaction after dilution of NH_2OH, could account for the apparent delay in the onset of the inactivation process (Fig. 6). Such a repair process would presumably depend on the concentration of liberated manganese, e.g.,

$$NH_2OH + Mn \cdot C \rightleftharpoons NH_2OH \cdot Mn \cdot C \rightarrow NH_2OH + Mn^{+2} + C^* \qquad \text{(extraction)}$$

$$Mn^{+2} + C^* \rightarrow Mn \cdot C \qquad \text{(repair)}$$

The repair process would become more rapid as the extraction process proceeds. Consequently the presence of the repair process during the assay procedure would lead to a lengthened kinetic for the O_2-evolution measurements with respect to that for the R_1 measurements as observed in Fig. 6. It should also be noted, by the same reasoning, that the time-course for the appearance of enhanced relaxivity probably reflects two opposing reactions and thus does not measure strictly the time-course of the forward (extraction) reaction.

Successful reconstitution of high rates of oxygen evolution following NH_2OH extraction of Class II chloroplasts has not previously been reported, although reconstitution in whole algal cells has been observed following illumination [20,21,53]. The data of Fig. 4 suggest that repair of oxygen-evolving centers in chloroplasts may occur naturally in the dark under appropriate conditions. An investigation of this phenomenon may assist in elucidating the mechanism by which NH_2OH inactivates the oxygen-evolving reaction in PS II.

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