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On the action of hydroxylamine, hydrazine and their derivatives on the water-oxidizing complex

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Abstract. Photosynthetic water oxidation proceeds by a four-step sequence of one-electron oxidations which is formally described by the transitions $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow (S_4) \rightarrow S_0$. State S_1 is most stable in the dark. Oxygen is released during $S_3 \rightarrow (S_4) \rightarrow S_0$. Hydroxylamine and hydrazine interact with S_1. They cause a two-digit shift in the oxidation sequence as observed from the dark equilibrium, i.e. from $S_1 \rightarrow S_2$: $S_2 \rightarrow S_3$: $S_3 \rightarrow (S_4) \rightarrow S_0$: $S_0 \rightarrow S_1$: . . . in the absence of the agents, to $S_1^* \rightarrow S_0$: $S_0 \rightarrow S_1$: $S_1 \rightarrow S_2$: $S_2 \rightarrow S_3$: . . . in the presence of hydroxylamine or hydrazine.

We measured the concentration dependence of this two-digit shift via the pattern of proton release which is associated with water oxidation. At saturating concentrations hydroxylamine and hydrazine shift the proton-release pattern from $OH^+(S_1 \rightarrow S_2) : 1H^+(S_2 \rightarrow S_3) : 2H(S_3 \rightarrow S_0) : 1H^+(S_0 \rightarrow S_1) : \ldots$ to $2H^+(S_1^* \rightarrow S_0) : 1H^+(S_0 \rightarrow S_1) : OH^+(S_1 \rightarrow S_2) : 1H^+(S_2 \rightarrow S_3) : 2H^+(S_3 \rightarrow S_0) : \ldots$ The $2H^+$ were released upon the first excitation with a half-rise time of 3.1 ms, both with hydroxylamine and with hydrazine. The concentration dependence of the shift was rather steep with an apparent Hill coefficient at half saturation of 2.43 with hydroxylamien (Förster and Junge (1985) FEBS Lett. 186, 53–57) and 1.48 with hydrazine. The concentration dependence could be explained by cooperative binding of $n \geqslant 3$ molecules of hydroxylamine and of $n \geqslant 2$ molecules of hydrazine, respectively. Tentatively, we explain the interaction of hydroxylamine and hydrazine with the water-oxidizing complex (WOC) as follows: Two bridging ligands, possible Cl^- or OH^-, which normally connect two Mn nuclei, can be substituted by either 4 molecules of hydroxylamine or 2 molecules of hydrazine when the WOC resides in state S_1.

Abbreviations

DNP–INT, dinitrophenylether of iodonitrothymol; FWHM, full width at half maximum; NR, neutral red (3-amino-7-dimethylamino-2-methyl-phenazine·HCl); PS II, photosystem II; WOC or (in formulas:) W, water-oxidizing complex.

Dedicated to Prof. L.N.M. Duysens on the occasion of his retirement.

Introduction

When dark-adapted thylakoids are excited by a series of single-turnover flashes of light, water is oxidized in a four-step sequential process which is formally described by the transitions $S_1 \rightarrow S_2$ (S_1 stable in the dark), $S_2 \rightarrow S_3$, $S_3 \rightarrow S_4 \rightarrow S_0$, $S_0 \rightarrow S_1, \ldots$, [13]. Dioxygen is liberated during $S_3 \rightarrow S_4 \rightarrow S_0$. Hydroxylamine ($NH_2OH$), hydrazine ($NH_2NH_2$) and certain derivatives of these molecules interact with state S_1 in the dark. We call the resultant modified state S_1^*. (This formal assignment does not infer that the redox state of the WOC is the same in S_1 and S_1^*.) Illumination then induces the oxidation sequence $S_1^* \rightarrow S_0$, $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_0, \ldots$ This two-digit shift was observed via the pattern of oxygen evolution [1, 16] and via the pattern of proton release [8] associated with the water-oxidation cycle. The stoichiometric pattern of proton release (into the thylakoid lumen) is shifted from $0H^+(S_1 \rightarrow S_2): 1H^+(S_2 \rightarrow S_3): 2H^+(S_3 \rightarrow S_4): 1H^+(S_0 \rightarrow S_1: \ldots$ (unmodified) to $2H^+$ (first flash): $1H^+(S_0 \rightarrow S_1): 0H^+(S_1 \rightarrow S_2): 1H^+(S_2 \rightarrow S_3) \ldots$ in the presence of NH_2OH or NH_2NH_2.

The mechanism of the action of these agents is not understood. The water-oxidizing complex is supposed to be a binuclear or tetranuclear manganese complex [3]. It has been speculated by Radmer and Ollinger [16, 17] that NH_2OH and NH_2NH_2 act as 'water analogues', i.e. that they substitute the oxygen precursor, $2H_2O$, at the active site of the complex. Previously, we studied the concentration dependence of the two-digit shift with the aim to determine the coordination number of hydroxylamine to the WOC. We found a cooperative action of at least 3 molecules of NH_2OH [9]. Here, we present new studies on the action of NH_2OH, NH_2NH and derivatives of these molecules. In the light of the present results we discuss the implications for structure and function of the WOC.

Materials and methods

Thylakoids were prepared from peas (Pisum sativum), frozen and stored under liquid nitrogen until use [5, 7].

pH changes in the thylakoid lumen were monitored via absorption changes of neutral red (± 13 μM NR), the external phase being buffered by 1.3 g/l bovine serum albumin (for the method see [11, 12]). Photometric measurements were carried out as described in detail in [7].

Hydroxylamine hydrochloride (H-9876), hydrazine dihydrochloride (H-6628), *O*-methylhydroxylamine hydrochloride (M-1139) and *O*-sulfonylhydroxylamine (H-0134) were purchased from Sigma and dried under vacuum before use. *N*-methylhydroxylamine hydrochloride was purchased from Merck (No. 820802). In stock solutions of these salts hydrochloride was neutralized by KOH. They were used for up to 10 h at longest.

For measurements, thylakoids were thawed and diluted in the dark to a

final concentration of 20 μM chlorophyll. The medium contained 2 mM potassium hexacyanoferrate(III), 25 mM KCl, 3 mM $MgCl_2$, 5 μM 2,6-dimethyl-p-benzoquinone (pH 7.2); 4 μM DNP–INT was present in all samples in order to abolish internal pH transients due to photosystem I [7]. The complete suspension was kept in the dark at room temperature for at least five minutes before measurement. A given dilute suspension was used for 10–20 min.

Results

Modification of the proton-release pattern associated with water oxidation by different 'water analogues'

Figure 1, trace a shows the typical pattern of proton release by PS II (DNP–INT present) into the thylakoid lumen which is observed upon excitation of dark-adapted thylakoids with a series of short flashes (compare [7]). The rapid pH jumps which are not time resolved in this particular measurement (half-rise time < 2 ms) exhibit a damped periodical pattern as function of flash number (compare Figure 2a). In terms of S-state transitions this is interpreted by the release per PS II of $OH^+(S_1 \rightarrow S_2)$: $1H^+(S_2 \rightarrow S_3)$: $2H^+(S_3 \rightarrow S_4 \rightarrow S_0)$: $1H^+(S_0 \rightarrow S_1)$ . . . [7, 10]. Since we have to assume that slower phases are not due to water oxidation [7] we shall use the term 'proton yield' only for the fraction of absorption changes with half-rise times of less than 2 ms.

For the sake of reproducibility aliquots of large stocks of homogeneous frozen thylakoids (2–4 mM Chl) were used for all experiments presented in previous papers [6–9] as well as in this study. It should be noted that the kinetics of the internal pH changes (repetitive excitation [5]) as well as the pattern of proton release (Förster, unpublished) are the same independently of whether the starting material is freshly prepared or frozen, provided that 30% ethylene glycol has been present in the freezing medium.

Figure 1, traces b–f show the proton-release pattern measured in the presence of 'water analogues'. We limited the measurements to concentrations at which the compounds did not destroy more than 10% of the water-oxidizing centers irreversibly (tested via oxygen evolution rates at continuous illumination after washing out these agents by a single centrifugation step). 14 μM NH_2OH, 60 μM NH_2OSO_3H and 120 μM NH_2NH_2 (Figure 1, traces b, c, f and Figure 2b, c) caused a two-digit shift in the proton-release pattern, which then was $2H^+$ (first flash): $1H^+(S_0 \rightarrow S_1)$: $OH^+(S_1 \rightarrow S_2)$: $1H^+(S_2 \rightarrow S_3)$: $2H^+(S_3 \rightarrow S_4 \rightarrow S_0)$ . . . (compare Figure 2a, b, for hydroxylamine see also [9]). 500 μM CH_3–NHOH (Figure 1, trace e) had only a moderate effect, and 250 μM NH_2–O–CH_3 (Figure 1, trace d) had no effect on the proton-release pattern.

With NH_2OH, NH_2OSO_3H and NH_2NH_2 present at saturating concentrations proton release upon the first flash was time resolved (Figure 3). With

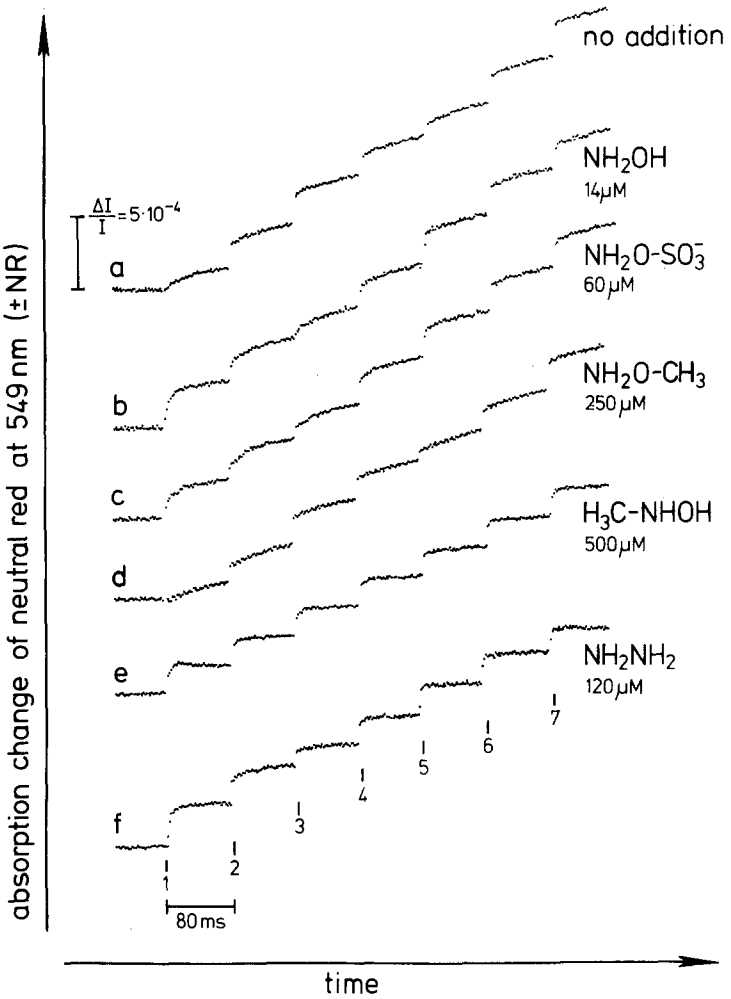

Figure 1. Absorption changes of neutral red at 549 nm (± NR) in response to a series of saturating flashes, measured in the absence and in the presence of NH_2OH, NH_2OSO_3H, NH_2OCH_3, CH_3NHOH and NH_2NH_2 at concentrations as indicated. Flashes were provided by a PRA 610B Xenon flash lamp (2 μs FWHM), the measuring light intensity was 90 μW at 548 nm.

each of these agents the same proton yield at the same half rise time was found: $2H^+$ at 3.1 (± 0.5) ms. We will discuss this phenomenon below. (The difference between this half-rise time and that published previous for NH_2OH (1.7 ms in [8]) is explained by the following: This kinetic component is followed by a slow drift which is supposedly not due to water oxidation [7, 8]. With increasing concentration of amines the slow drift vanished. In Figure 3 it is virtually absent, which allowed a more accurate determination of the half rise time from these traces than formerly, where the rapid phase was apparently faster due to superposition of the slow drift.)

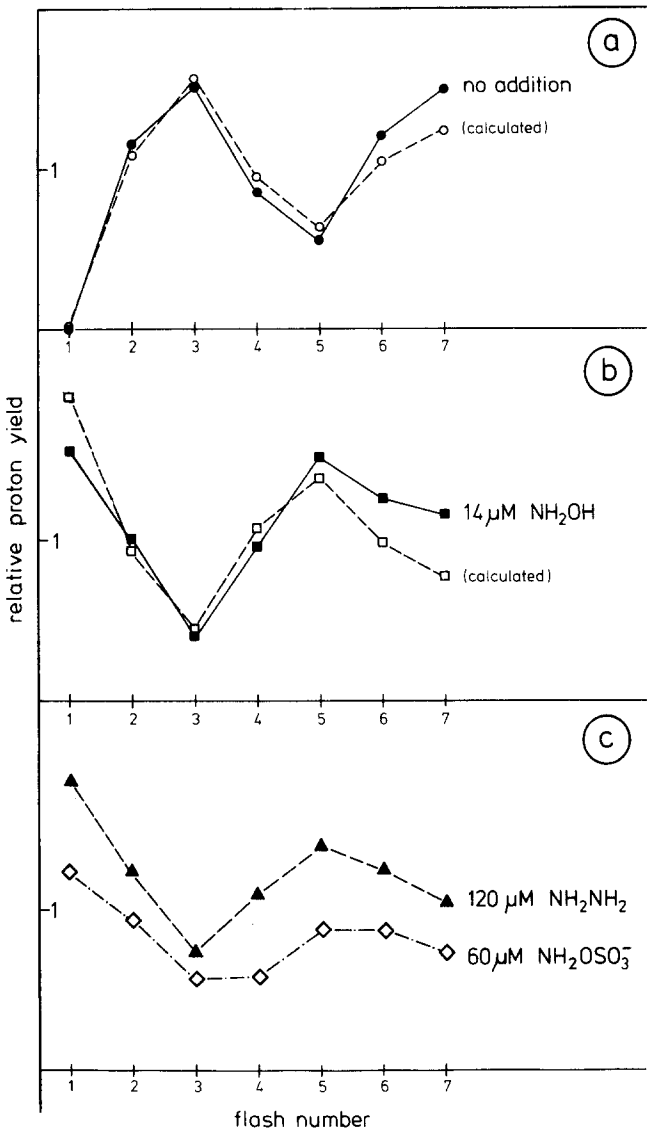

Figure 2. Amplitudes of internal pH changes of $\tau < 2$ ms half-rise time as function of flash number, as obtained by kinetic analysis of the traces in Fig. 1; additives as indicated. Open symbols in (a) and (b): patterns calculated under the assumption of 100% S_1 (a) and 100% S_1^* (b) in the dark, 10% misses and 10% double hits.

Concentration dependence of the two-digit shift of the proton-release pattern by water analogues

Previously, we studied the concentration dependence of the two-digit shift of the proton-release pattern as effected by NH_2OH [8, 9]. We find that the

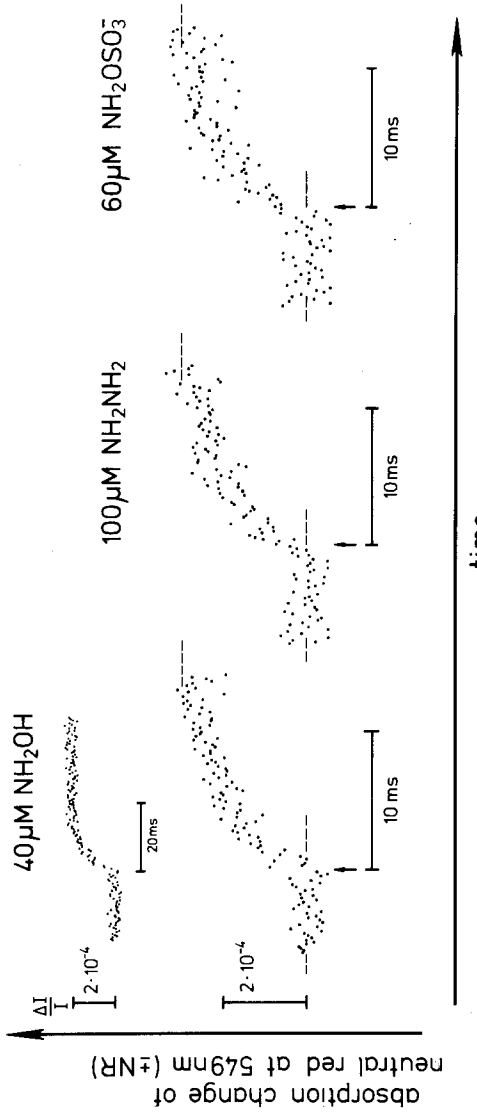

Figure 3. Time-resolved absorption changes of neutral red at 549 nm (± NR) upon the first flash given to thylakoids which had been incubated in the dark for at least five minutes with 40 μM NH_2OH, 100 μM NH_2NH_2 and 60 μM NH_2OSO_3H, respectively.

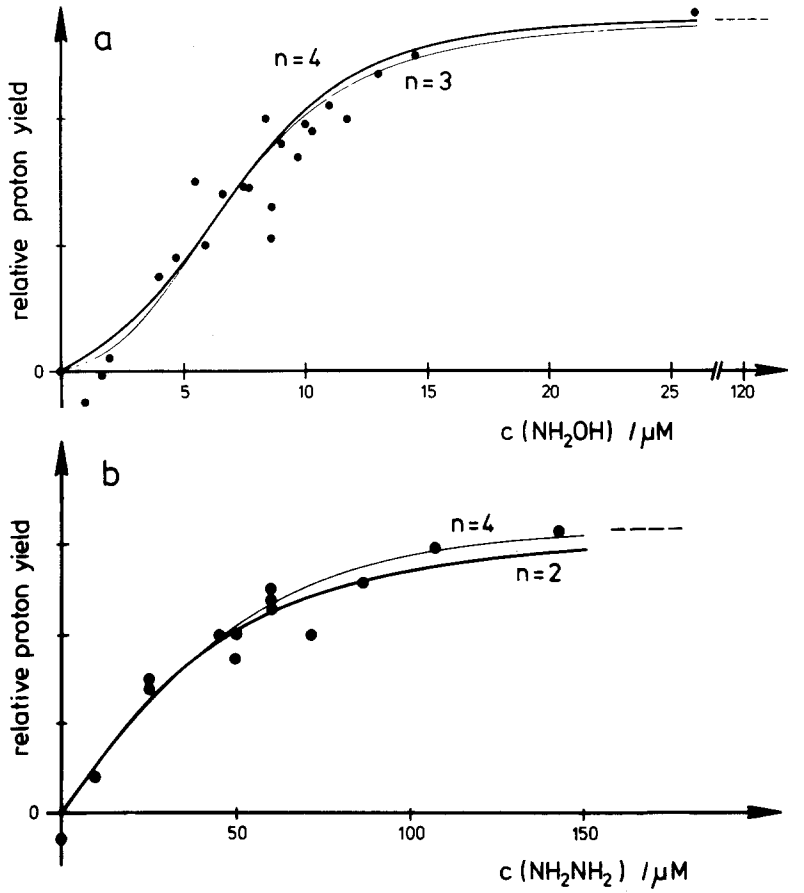

Figure 4. Proton yields upon the first flash as function of the concentration of hydroxylamine (a) and hydrazine (b), (Figure 4a taken from [9]). The solid curves were calculated under assumption of cooperative binding of 3 and 4 molecules of hydroxylamine, and of 2 and 4 molecules of hydrazine (see text).

concentration dependence (for comparison shown in Figure 4a) is rather sharp with an apparent Hill coefficient of 2.43 at half saturation. A quantitative fit (see lines in Figure 4a) is obtained by the model of sequential interaction of allosteric enzymes under the following assumptions: There are four allosterically interacting coordination sites for hydroxylamine at the WOC. The dissociation constant is $K_d = 100\,\mu M$ and the allosteric interaction factor from one state to the state of next higher occupation is $a = 1/5$. The proton-release pattern is one and the same no matter whether the WOC is loaded with 1, 2, 3 or 4 NH_2OH molecules and it is independent of their particular distribution of over the four sites [9].

In the present study, we investigated the concentration dependence of two-digit shift of the proton-release pattern as caused by NH_2NH_2. As in the

case of hydroxylamine, the pattern could be considered as the sum of the $(0:1:2:1:)_n$: ... and the $2:1:(0:1:2:1:)_n$: ... pattern at different percentages, depending on the concentration (data not shown). Figure 4b shows the rise of the first-flash yield as function of NH_2NH_2 concentration. The proton yields at zero concentration upon the first flash, $Y_1(c = 0)$, as well as the respective asymptotic yields at high concentration, $Y_1(\infty)$ were marked by dashed lines. The percentage, s, of water-oxidizing complexes which gives rise to the 'shifted' pattern of proton liberation, $2:1:(0:1:2:1:)\ldots$, is

$$s(c) = \frac{Y_1(c)}{Y_1(\infty)}$$

From a Hill plot, $\log(s/1-s)$ versus $\log c$, we obtained an apparent Hill coefficient at half saturation of 1.48 for NH_2NH_2, (compare 2.43 for NH_2OH [9]). The concentration at half saturation with NH_2NH_2 was 34 μM, higher than with NH_2OH (7 μM); (for the solid lines in Figure 4 see Discussion). A relative decrease of the yield at the third flash paralleled the relative increase of the yield at the first flash with the same concentration dependence (for NH_2OH see [9], for NH_2NH_2 not shown).

We also measured the proton-release pattern at various concentrations of NH_2OSO_3H. It turned out that the effect was always saturated at 60 μM NH_2OSO_3H. At intermediate concentrations, however, results varied considerably. In aqueous solution NH_2OSO_3H is known to hydrolyse in the hour time range, in the presence of nucleophilic agents even faster [14]. Thus, it is conceivable that in a thylakoid suspension NH_2OSO_3H hydrolyses within some minutes. Therefore, we suspect that the active species in this case was not hydroxylamine-O-sulfonic acid but its hydrolysis product NH_2OH.

Decomposition of the hydroxylamine-loaded complex and rebinding of hydroxylamine

When NH_2OH is added to dark-adapted thylakoids, it primarily reacts with state S_1 of the WOC. When, after 5–10 min incubation, thylakoids are submitted to a series of flashes they undergo the transitions $S_1^* \cdot nNH_2OH \rightarrow S_0$, $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2, \ldots$ [1, 8]. We studied the turnover time of the reaction $S_1^* \cdot nNH_2OH \rightarrow S_0$ by measuring the proton-release pattern at diminished dark times between the first and second flash. While the interval between the first and second flash was varied, the intervals between the following flashes was kept constant at 80 ms. With and interval of 30 ms between the first and second flash we found the centers only partially relaxed to the supposed S_0 state. (We could not determine this accurately since the proton-release pattern at the 30 ms interval was not just the sum of the '80 ms pattern' and the undisturbed pattern). In consequence, proton release upon

the first flash ($\tau_{1/2} = 3.1$ ms) resulted from an early product of $[S_1^* \cdot NH_2OH]^+$ while the native state S_0 was reached only at dark times greater than 30 ms.

We studied the recovery of the NH_2OH-modified proton-release pattern as function of the dark time between a group of two conditioning flashes (30 ns FWHM, flash distance 100 ms) and a subsequent series of probing flashes (spaced 80 ms apart). Except for a deviation upon the first probing flash, the probing flash series evoked a proton-release pattern which was similar to the pattern observed in NH_2OH-free thylakoids. This was independent of the dark time between the conditioning flashes and the probing flashes in the range from 80 ms to 10 s. No rebinding seemed to occur in this time range. This is compatible with observations made on a longer time scale by other investigators [19]. The deviation consisted in an additional proton yield ($\tau_{1/2} < 1$ ms) upon the first probing flash.

Discussion

Classical biochemical methods in the investigation of enzymatic mechanisms take advantage of the variation of substrate concentration or of substituents of the substrate. Since in photosynthetic oxygen evolution H_2O is solvent as well as substrate these methods fail. It has been hoped to gain insight into the mechanism of water oxidation via application of 'water analogous' artificial electron donors. It has been expected that these artificial donors bind in competition with water to the WOC, which is assumed to be a manganese complex with a 2(+ 2) arrangement of the metal nuclei.

H_2N–OH, H_2N–NH_2 and some derivatives shift the phase of the period-of-four oscillation of water-oxidation under a series of exciting flashes applied to the dark-adapted material from $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow (S_4) \rightarrow S_0$, $S_0 \rightarrow S_1$, ... to $S_1^* \rightarrow S_0$, $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, ... [1, 7, 16]. We investigated this two-digit shift as reflected in the proton-release pattern (Figure 1, 2). In their respective concentration ranges of reversible interaction with the WOC, NH_2OH and NH_2NH_2 caused a complete two-digit shift. The hydroxylamine derivative CH_3–NH–OH was only moderately effective. Among the *O*-substituted hydroxylamine derivatives, the hydrophobically substituted compound H_2N–O–CH_3 was entirely ineffective. The hydrophilically substituted compound H_2N–O–SO_2H, on the other hand, was effective (compare [18]). However, the effective species in the latter case may be the hydrolysis product NH_2OH (see above). Thus, it appeared that the interaction with the WOC was restricted to those molecules which did not or only little exceed the sterical dimensions of the NH_2OH molecule.

The similarity between NH_2OH, NH_2NH_2, ... and two H_2O suggested to consider these molecules as competitive substrates at the active site of the WOC, as outlined by Radmer and Ollinger [1, 17]. We obtained the number of interacting molecules from the concentration dependence of the two-digit shift of the water-oxidation cycle effected by NH_2OH and NH_2NH_2.

From the Hill coefficients n at half saturation it was directly evident that $\geqslant 3$ NH_2OH molecules (n = 2.43) or $\geqslant 2$ NH_2NH_2 molecules (n = 1.48) interacted with the WOC in the dark. In [9] we fitted the effect of NH_2OH by a sequential interaction model for multi-site enzymes. It is worthwhile to summarize briefly this model before discussing the results in the light of the literature on water oxidation and coordination chemistry.

The sequential interaction model (see for instance section 4 in [20]) considers the following equilibria:

$$W \underset{K_d}{\overset{+D}{\rightleftharpoons}} WD_1 \underset{aK_d}{\overset{+D}{\rightleftharpoons}} WD_2 \underset{bK_d}{\overset{+D}{\rightleftharpoons}} WD_3 \underset{cK_d}{\overset{+D}{\rightleftharpoons}} WD_4 \rightleftharpoons \ldots$$

W = water-oxidizing complex;
K_d = dissociation constant;
a, b, c = sequential interaction factors;

The water-oxidizing complex had n equivalent binding places. The degeneracy of the states WD_i followed the pattern:

(1, 2, 1) for n = 2

(1, 3, 3, 1) for n = 3

(1, 4, 6, 4, 1) for n = 4

With these assumptions the following 'normalized reactivities' s_n, i.e. the percentages of water-oxidizing centers modified in proton liberation, for enzymes with 2, 3 and 4 sites were:

$$s_2 = (2X + X^2/a)/(1 + \text{nominator})$$

$$s_3 = (3X + 3X^2/a + X^3/a^2b)/(1 + \text{nominator})$$

$$s_4 = (4X + 6X^2/a + 4X^3/(a^2b) + X^4/(a^3b^2c))/(1 + \text{nominator})$$

$X = [D]/K_d$ = effector concentration, [3], as normalized to the dissociation constant, K_d;

It was assumed that each of the ligands was equally effective but that the effect was the same independent of the number of ligands coordinated to the WOC.

Coordination of 'water analogues' to the water-oxidizing complex

Assuming that the sequential interaction factors were equal (a = b = c), we obtained fits to the experimental points with several sets of parameters (listed in Table 1). In Figure 4 these fits are shown by solid lines. Although the fits obtained with coordination numbers of 3 and 4 for NH_2OH and of 2 and 3 for NH_2NH_2 were not distinguishable at the experimental resolution we considered binding of 4 NH_2OH or of 2 NH_2NH_2 as most probable, since they required only a moderate allosteric interaction factor (a = 1/5). This

Table 1. Fit parameters for the concentration dependence of the two-digit shift of the water-oxidation cycle by NH_2OH and NH_2NH_2

Compound	Number of bound molecules	K_d	Allosteric factor a = b = c
NH_2NH_2	2	117 μM	0.2
	3	232 μM	0.05
NH_2OH	3	152 μM	0.05
	4	97 μM	0.2

factor decreased the binding constant from 100 μM, valid for the first ligand, to 800 nM for the fourth ligand. If the allosteric factor was a = 1/20, as assumed for the alternative fit curves, the binding constant would have been reduced from 100 μM to 12.5 nM. For comparison it may be noted that the allosteric interaction factor of hemoglobin (4 binding sites, apparent Hill coefficient 2.8) is in the moderate range (a = 1/6.7). Despite of these arguments, and if one considers that manganese is dimeric or tetrameric in the WOC [3], uneven coordination numbers are unlikely. Therefore, we assume that the WOC can bind up to 4 NH_2OH or up to 2 NH_2NH_2, respectively.

Implications for the structure of the water-oxidizing complex

Coordination of 4 NH_2OH to the WOC would imply 4 or 8 binding sites on manganese, depending on whether it reacted as a mono- or as a bidentate ligand. While NH_2OH may act as chelating ligand NH_2NH_2 usually does not so, it rather bridges two metal centers [15]. Assuming that NH_2NH_2 occupied the same binding sites as NH_2OH we arrived at the following tentative structural model for a two-manganese complex (Figure 5): In the undisturbed complex two manganese are ligated by a bridging ligand (Figure 5A). NH_2NH_2 replaces the bridging ligand which leads to a distorted complex with enlarged Mn–Mn distance. Structural elements as in Figure 5B have been found in $[Mn(NH_2NH_2)_2Cl_2]_n$ crystals [4]. Similarly, NH_2OH might break the metal–metal bridges (Figure 5C). The bridging ligand replaced by NH_2OH and NH_2NH_2 may be Cl^- or OH^-. In the case of OH^- this may result in a displacement of the O_2 precursor. The two protons observed upon the first flash in the presence of NH_2OH or NH_2NH_2 as well as the two protons observed during $S_3 \rightarrow S_0$ may be due to the insertion of new bridging ligands after release of NH_2OH, NH_2NH_2 or O_2, respectively, according to $2H_2O \rightarrow 2OH^-_{bound} + 2H^+$.

Radmer and Ollinger [17] speculated on the size of the catalytic site of the WOC based on the assumption of a structural and functional analogy between NH_2OH and the O_2 precursor. Since they assumed that the reactive site was occupied by only one molecule of NH_2OH they postulated a minimum size of 2.5 × 4 Å. In the light of the coordination of 4 NH_2OH to the WOC, however, a functional analogy is questionable. Contrary to Radmer's and

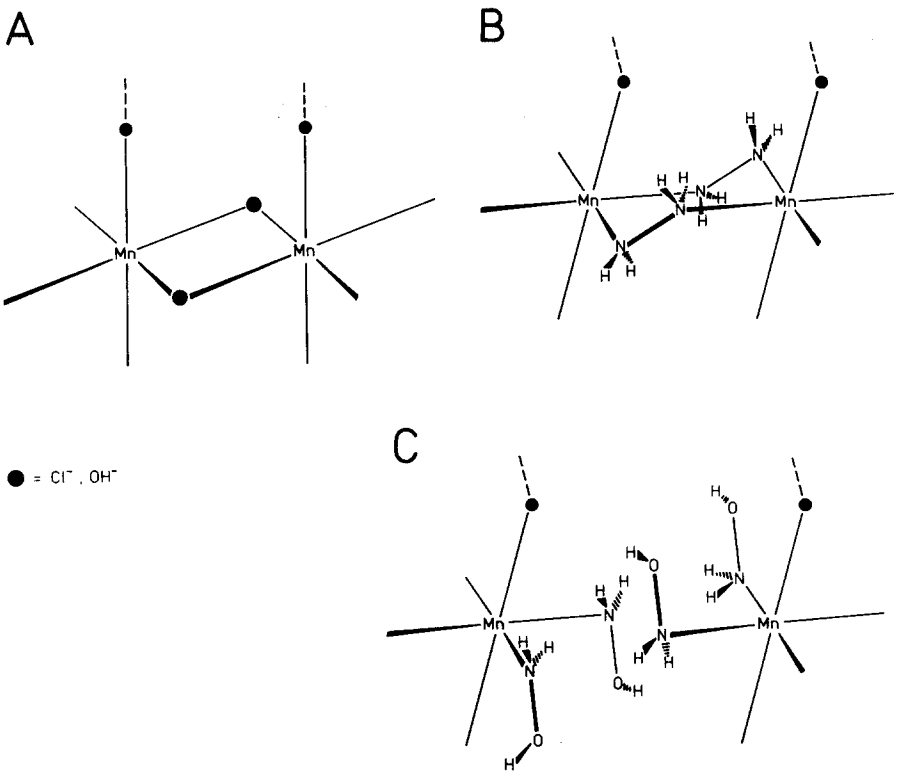

Figure 5. Hypothetical structural elements of the WOC. A: in native state S_1; B and C: after reaction with NH_2NH_2 and NH_2OH, respectively; (for explanation see text).

Ollinger's assumption of a rigid conformation, the binding of four molecules NH_2OH entails conformational changes in the protein. In the hypothetical complex shown in Figure 5 the Mn–Mn distance of the native chloride-bridged complex A is ≈ 3.8 Å. It has to be enlarged to 4.5 Å for the binding of 2 NH_2NH_2 (B) and to > 5.5 Å for the binding of NH_2OH (C). The relatively long time which is needed for relaxation to the S_0 state after the first flash (note, that 30 ms were insufficient) may be due to the displacement of NH_2OH and to the reversal of conformational changes.

Reactivity of the hydroxylamine-loaded complex

It is remarkable that with either compound, NH_2OH and NH_2NH_2, we found a monophasic release of two protons per PS II reaction center with a half rise time of 3.1 ms. This half-rise time is longer than those observed during regular water oxidation. Since neither the amount nor the rate of proton release depended on the chemical nature of the 'water analogue' it is reasonable to assume that the first flash causes the oxidation of the respective bound 'water analogue', but that the proton release resulted from a secondary

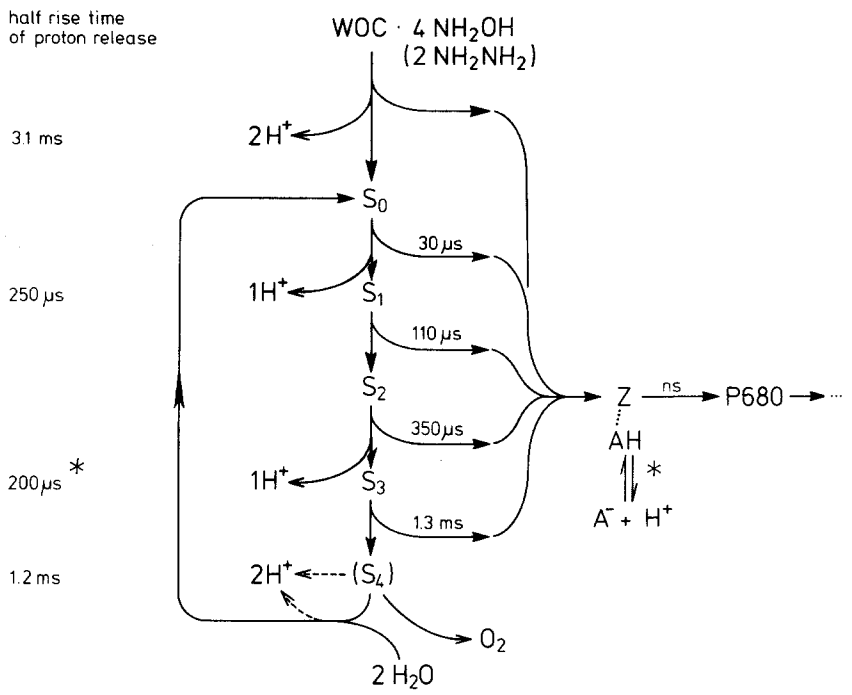

Figure 6. Scheme summarizing electron transfer and proton release at the donor side of PS II, including modification by hydroxylamine and hydrazine.

reaction of the WOC. This secondary reaction might be the reinsertion of water after ejection of the NH_2OH-oxidation products according to $2H_2O_{free} \rightarrow 2H^+ + 2OH^-_{bound}$ in a state which is precursor to S_0.

Synopsis

The scheme of Figure 6 may help to summarize our present understanding of photosynthetic water oxidation. By successive one-electron reactions via an intermediate electron carrier, Z, the water-oxidizing complex is oxidized from state S_0 to the higher oxidation states S_1, S_2, S_3 and S_4 at reaction half-rise times between 30 µs to 1.3 ms, increasing with the oxidation state [2] (Fig. 6). State S_4 spontaneously relaxes to S_0, thereby releasing O_2. Proton release is associated with the transitions $S_0 \rightarrow S_1$ ($1H^+$, 250 µs), $S_2 \rightarrow S_3$ ($1H^+$, 200 µs) and $S_3 \rightarrow (S_4) \rightarrow S_0$ ($2H^+$, 1.2 ms) [7]. The chemical nature of the protolytic reactions is unknown. However, differentiations can be made by comparison of the half-rise time of proton release with the kinetics of electron abstraction from the water-oxidizing complex. While proton release during $S_0 \rightarrow S_1$ and during $S_3 \rightarrow S_4 - S_0$ appears to occur on the level of the water-oxidizing complex, an additional proton push and pull coupled to oxidoreduction of Z has to be assumed in order to explain the

200 μs component of proton release during $S_2 \rightarrow S_3$ (marked by an asterisk in Figure 6) [7].

In the dark, 1–4 molecules of NH_2OH and 1 or 2 molecules of NH_2NH_2 cooperatively bind to the WOC. Abstraction of only one electron apparently leads to state S_0 [8]. This is accompanied by release of $2H^+$ with $\tau_{1/2} = 3.1$ ms, independently of the compound. Complete relaxation to S_0 obviously takes considerably longer (30 ms were insufficient). We suggest that NH_2OH and NH_2NH_2 act on state S_1 by displacing bridging ligands between two manganese centers under enlargement of the Mn–Mn distance and under conformational changes in the protein, which both should be measurable in further work.

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