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Protolytic Reactions of the Photosynthetic Water Oxidase in the Absence and in the Presence of Added Ligands

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Abstract

Photosynthetic water oxidation proceeds by a sequence of four one-electron abstractions by photosystem II (PS II) from the catalytic manganese centre, which accumulates four oxidizing equivalents. This is formally described by transitions $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, and $S_3 \rightarrow S_1 \rightarrow S_0$, with state S_1 most stable in the dark. Dioxygen is produced and protons are liberated into the thylakoid lumen. Flash-spectrophotometric techniques have previously revealed the stoichiometry and kinetics of proton release per univalent transition.

Via the pattern of proton release as function of flash number we have studied the reactions of NH_2OH and of NH_2NH_2 as putative ligands and electron donors to the Mn centre. Our results suggest reversible binding of these agents to the centre in state S_1, completed after 5 min incubation. After excitation of PS II by a first flash of light a two-step reaction is initiated. The first step is apparent via the release of two protons into the lumen with approximately 3 ms half-rise-time. The second, with 30 ms, is apparent by relaxation of the centre into state S_0. The effect of NH_2OH is not influenced by chloride in the concentration range between 100 μM and 30 mM. This excludes competitive binding of hydroxylamine to chloride-binding sites. The concentration dependence is very steep with Hill coefficients 2.4 (NH_2OH) and 1.5 (NH_2NH_2). This can be understood in terms of co-operative binding to at least 3, if not 4, binding sites for hydroxylamine. If a binuclear manganese centre accommodated four molecules of hydroxylamine (bidentate complexes) or two molecules of hydrazine as bridging ligands, relatively large Mn-Mn distances were expected (up to 5.4 Å). These have not been observed in EXAFS. We show that the compared with optical studies, 100-fold more concentrated samples used in EXAFS are heterogeneous with regard to the occupation of centres with 0, 1, 2, 3, 4 molecules of hydroxylamine. They have therefore to be reinterpreted.

Introduction

The photosynthetic water oxidase is a complex enzyme which integrates a photochemical reaction centre, photosystem II (PS II), and a manganese cluster as catalytic centre (reviewed in refs. [1,2]). One quantum of light drives a very rapid charge separation, half rise time about 400 ps, between a donor chlorophyll *a*, P680, and a bound plastoquinone, Q_a. This reaction is directed from the lumenal side of the thylakoid membrane to the stroma side. It fully spans the membrane dielectric [3]. The manganese cluster acts as an accumulator of oxidizing equivalents which are produced one by one in PS II. Once four oxidizing equivalents are accumulated two molecules of water are oxidized to one molecule of dioxygen [4,5]. The step-by-step accumulation of oxidizing equivalents is formally described by transitions between five states, S_0 to S_4, where S_1 is most stable in the dark, and S_4 spontaneously decays to S_0 under liberation of dioxygen [6].

Because the catalytic manganese centre and the bound quinones are placed at different sides of the membrane the water oxidase acts as an electrogenic proton pump. The photochemical electron transfer charges the lumenal side positively. Water oxidation releases protons into the lumen, and quinone reduction is followed by proton uptake from the stroma. This generates a pH-difference [3]. It is unique to photosynthetic membranes that the elementary electrochemical reactions are measurable by flash spectrophotometric techniques at high time resolution [3]. Proton release into the thylakoid lumen, in particular, is a sensitive indicator of the sequential reactions of the water oxidase.

Water is substrate and solvent of the water oxidase. That its concentration cannot be varied hampers studies on its interaction with the catalytic site. Hydroxylamine and hydrazine interact with the water oxidase. They are of great interest under the aspect that they may bind to and react with the catalytic manganese centre proper. We investigated their highly co-operative binding to the oxidase.

Experimental

Thylakoids were prepared from pea seedlings as described in [14]. Concentrated stock suspensions were stored on ice until dilution of aliquots in a medium containing 25 mM-KCl, 3 mM-$MgCl_2$, 2.6 g/l bovine serum albumin, 4 μM-DNP-INT, 2 mM potassium hexacyanoferrate(III). Spectrophotometric experiments were carried out with the dilute suspension at 20 μM chlorophyll in a cuvette of 1 cm optical pathlength and at room temperature. PS II was excited with single turnover flashes of red light (Xenon, 1 μs or Ruby laser, 40 ns FWHM). pH transients in the lumen were detected photometrically by way of transient absorption changes of neutral red (13 μM), bovine serum albumin acted as selective buffer for the suspending medium [8]. The response of neutral red to pH transients resulted from a subtraction of a transient absorption change at a wavelength of 548 nm obtained in the absence of neutral red from another one obtained in its presence [8].

The submicroscopic dimensions of the lumen, radius about 300 nm and width about 5 nm, imply that there is only 0.1 free proton around at pH 7. Nevertheless is the pH in each thylakoid well defined as a time average with a standard deviation depending on the number of buffering groups

inside this volume, their protonation/deprotonation time and the time interval under inspection. To give an example: at pH 7 there are about 2×10^4 effective buffering groups in a thylakoid of the above dimensions (see ref. [8]) which according to Gutman's work on the kinetics of acid/base reactions at protein surfaces [9] undergo diffusion-controlled protonation. This implies a protonic on/off relaxation time in the order of 200 μs for each of these groups. Taken together they provide a protonic relaxation time of some 10 ns in the lumen. In other words the concentration of free protons when averaged over a time interval of 100 μs is precise within less than 3% error and the pH is defined within less than 0.02 units around pH 7.

Neutral red is sensitive for pH transients in the narrow lumen phase because of its membrane solubility. The free base has a distribution coefficient between membrane and aqueous bulk phase of 880 in favour of the membrane (ref. [10] in correction to ref. [11]). Neutral red, adsorbed to the membrane surface, is a true [8] indicator of the surface pH [11].

In response to the flash-induced pH difference, protonation of membrane-bound neutral red is very fast, for example 100 μs as found for proton release in Tris-treated thylakoids [7]. Redistribution of neutral red across the membrane occurs in the second time range and thus it only interferes with slower pH transients [10].

Results and discussion

Stoichiometry and kinetics of proton release during the sequential reactions of the water oxidase

The kinetic parameters of electron transport from the site of catalytic water decomposition via the intermediate carrier, Z, into P680 and the kinetics of concomitant proton release are summarized in Fig. 1. In dark-adapted pea thylakoids, the catalytic centre is predominantly in state S_1. Under excitation with a series of flashes the yield of rapid (< 3 ms) proton release proceeds as 0:1:2:1 per flash [12–14]. (The extent of rapid proton release after damping out of the period-of-four oscillations after many flashes is taken as standard, which, according to several lines of evidence, represents one proton per flash and PS II (α-centre) (reviewed in ref. [3]).) The lack of proton extrusion during transition $S_1 \rightarrow S_2$ leaves a positive net charge in the catalytic site which is not compensated until two protons are liberated during the transition $S_3 \rightarrow S_4$. The transients of the net charge, detected by Saygin and Witt [15] via electrochromic absorption changes, are consistent with this notion. The rate of electron transfer between Z and $P680^+$ is slowed down under conditions where the centre carries a positive net charge. This is attributed to coulombic attraction of the electron by the net charge in the centre [16].

In brief, the catalytic centre of the water oxidase acts as an accumulator of four oxidizing equivalents. Its net charge is controlled by the release of protons into the thylakoid lumen. During the four transitions $S_0 \rightarrow S_1$ to $S_3 \rightarrow S_4 \rightarrow S_0$ the extent of proton release oscillates like 1:0:1:2 protons per flash. This leaves a net charge of $0(S_0):0(S_1):+1(S_2):+1(S_3)$ in the catalytic centre.

The ultimate source of liberated protons is the oxidation of water. Transient proton release after a distinct flash, however, does not necessarily reflect the oxidation of 'bound water'. This becomes obvious by comparison of the half-rise time of electron transfer between the catalytic site and Z [17, 18] and the half-rise time of proton liberation [14]. Proton release follows in time electron abstraction from the catalytic site with one exception (see Fig. 1); in $S_2 \rightarrow S_3$ the half-rise time of proton release (200 μs [14]) is shorter than the one of electron abstraction from the catalytic site (350 μs [18]). In consequence, when the catalytic centre is in state S_2 proton release at 200 μs has to be attributed to a transient deprotonation/protonation of the intermediate electron carrier Z or of an acidic group in its vicinity [14].

We consider it as likely that protons are transiently liberated from amino-acid side groups which are only reprotonated during the reaction of the catalytic centre with water.

How to get hold of the so far ill-defined groups involved in the storage and channelling of protons? A possible clue might be expected from work by Peter Jahns and Andrea Polle in our laboratory. They found the proton-pumping activity of PS II short-circuited after covalent modification by *N,N'*-dicyclohexylcarbodiimide of two particular polypeptides in the range of 20–24 kD molecular mass. Treatment with this notorious blocker of proton channels indeed blocked proton release and also proton uptake but it also opened a channel which conducted protons, produced during water oxidation, across the membrane to the freshly reduced quinone. It will be interesting to identify the respective polypeptides among the about 15 ones [19] which are contained in the photosynthetic water oxidase.

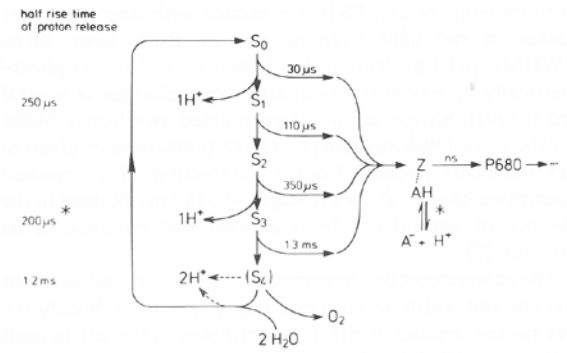

Fig. 1. Electron transfer and protolytic reactions at the donor side of PS II (for refs. see text).

The reaction of hydroxylamine and of hydrazine with the water oxidase: kinetics, binding parameters, structure and products

Bouges [20] was the first to report that hydroxylamine shifts the pattern of oxygen release as function of flash number by two digits to higher flash numbers. This 'delay' of the water oxidase is also seen in the pattern of proton liberation [23]. For hydroxylamine it is documented in Fig. 2. Upon flash no 1 two protons per PS II are released with a half-rise time of 3.1 ms. The following flashes produce the usual pattern oscillating with period of four but as if starting from state S_0 upon flash no. 2 [23]. Thus the maximum of oxygen evolution and of proton release is shifted from flash no. 3 in the absence to flash no. 5 in the presence of hydroxylamine

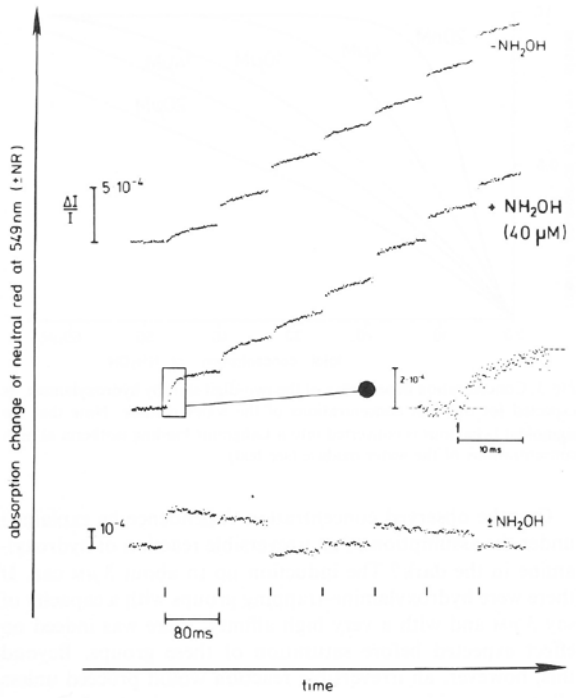

Fig. 2. Pattern of proton release into the thylakoid lumen observed upon a series of flashes in dark-adapted thylakoids, monitored by neutral red, without and with hydroxylamine (upper and middle trace). The bottom trace represents the difference between the middle and the upper trace. Time resolution of the pattern was 2 ms/point, and in the expanded region around the first flash of the middle trace 100 μs/point.

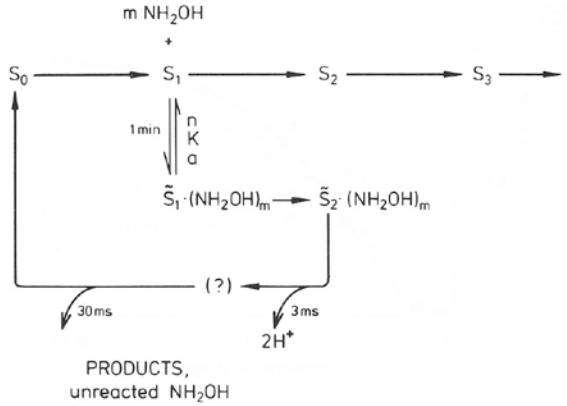

Fig. 3. Reactions of the water oxidase with hydroxylamine and their approximate half-rise times.

[20–24]. The same behaviour is observed in the presence of hydrazine and of *O*-sulphonyl-hydroxylamine [26].

It is under debate whether hydroxylamine reacts with the water oxidase already in the dark or whether it binds in the dark to react only after the centre is oxidized by input of one quantum of light. Judging from the pattern of proton release, we found that three washes of thylakoids in hydroxylamine-free medium abolished the effect of a previous incubation with hydroxylamine widely [23]. (Incubation plus washes were carried out all in total darkness!) This result was at variance from a report by Bouges who did not observe a reversal [20]. But it was in line with a report from Hanssum & Renger [24] who observed reversibility of the action for hydrazine. Thus we tend to assume that these agents bind reversibly to the centre in the dark (see also below and Fig. 3).

The kinetic parameters of hydroxylamine binding and its flash-induced reactions are given in Fig. 3. When dark-adapted thylakoids are incubated with 30 μM hydroxylamine the two-digit shift in the proton release pattern was fully expressed after 5 min, incubation for further 20 min did not enhance the effect. After firing of a single laser flash the release of two protons was half-completed in about 3 ms. If the second flash was fired 80 ms after the first one and a series of flashes at 80 ms intervals thereafter the pattern of proton release showed the period-of-four oscillation as documented in Fig. 2. The pattern was out of order, however, if the spacing between the first and the second flash was shorter. The time delay required for half-relaxation of the centre, supposedly to yield state S_0, was approximately 30 ms. Thus, we postulate two reaction steps following the first flash, one releasing protons and an about tenfold slower step completing the relaxation to S_0 (see Fig. 3). It is noteworthy that we found no protolytic reaction concomitant with or subsequent to the second reaction. This is documented in Fig. 2. The bottom trace is the difference between the proton release pattern in the presence and that in the absence of hydroxylamine. After flash no. 1 there is no extra proton produced or consumed except from the two which appear with 3 ms half-rise time (time resolution of the patterns was 2 ms/point).

In summary, the reaction of the water oxidase with hydroxylamine requires 5 min of incubation of dark-adapted thylakoids (at room temperature). One flash causes the release of two protons into the lumen at 3 ms half-rise time. The unperturbed state S_0 is reached with a half-rise time of approximately 30 ms. It is conceivable that this time interval is required for the release of products and of unreacted ligand molecules from the centre (see Fig. 3). Together with Friedhelm Lendzian in Klaus Möbius' laboratory we looked for an EPR-detectable intermediate. Unfortunately, no such intermediate could be detected in the time interval between 500 μs and some milliseconds after the flash. Either it was not produced, or it was too shortlived to be detected.

Which are the binding parameters of hydroxylamine and hydrazine to the catalytic centre? Figure 4*a* shows the extent of rapid proton release upon flash no. 1 in dark-adapted thylakoids as function of the hydroxylamine concentration (data points taken from ref. [25]). The effect *vs.* concentration behaviour is sigmoidal. A Hill plot reveals a Hill coefficient at half maximum of 2.43 for hydroxylamine (see Fig. 5 in ref. [5]) and of 1.48 for hydrazine [26]. For hydrazine the same degree of cooperativity was found by Hanssum and Renger [24]. At first sight this implies the existence of at least three binding sites for hydroxylamine and at least two for hydrazine, which are populated in a cooperative manner. A prerequisite for this interpretation is the reversible binding of these agents to the water oxidase. Before discussing the implications of the concentration dependence for the interaction of the agents with the catalytic centre in more quantitative terms we scrutinize alternative interpretations, in particular that of Radmer and Ollinger [21, 22] and of Beck and Brudvig (see this volume) who favour the idea that hydroxylamine irreversibly reacts with the water oxidase in state S_1 in the dark.

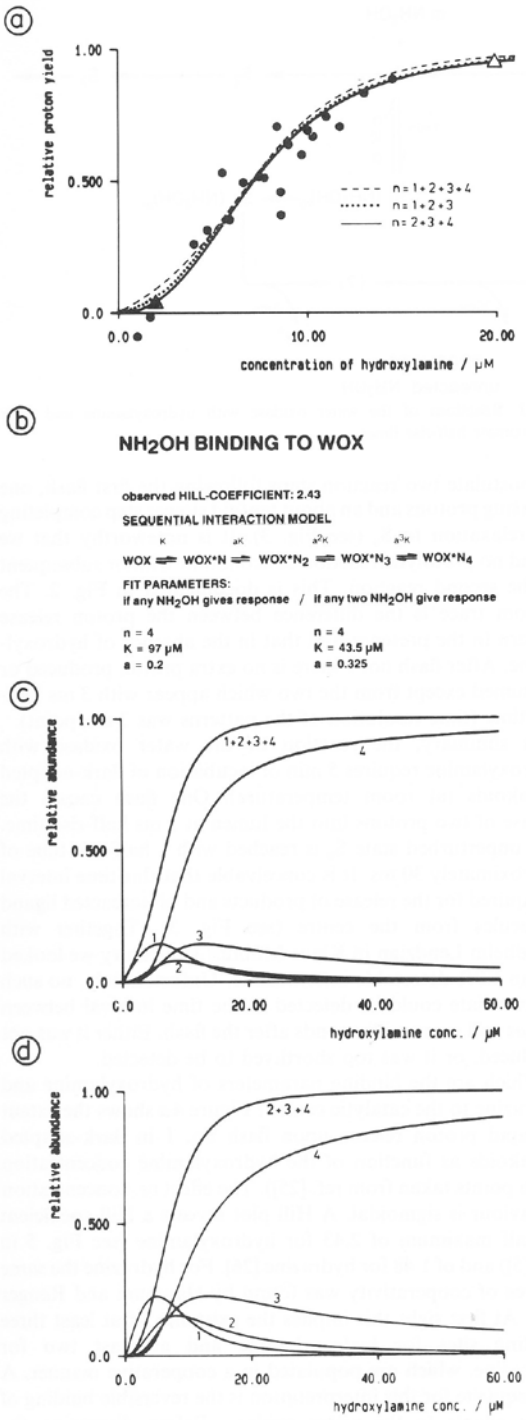

Fig. 4. Concentration dependence of the two-digit shift in the proton release pattern by hydroxylamine. (*a*) Hydroxylamine-mediated proton yield upon the first flash as a function of the concentration of hydroxylamine. Full circles: in the presence of 30 mM chloride. Open triangles: in the presence of 100 μM chloride. Lines: fit curves by model for sequential co-operative interaction (see Fig. 4*b* and text). (*b*) Reaction scheme for the co-operative reaction of the water oxidase with hydroxylamine and two parameter sets to fit the data in Fig. 4*a*. (*c*) and (*d*) Occupancy of states as function of hydroxylamine concentration for the two parameter sets (n, K, a) given in (*b*).

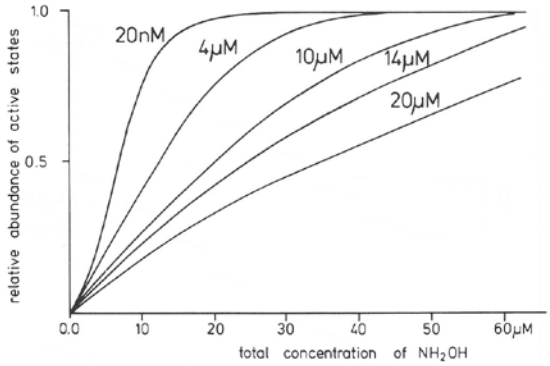

Fig. 5. Concentration dependence of the two-digit shift by hydroxylamine, as expected for different concentrations of the water oxidase. Note that the sigmoidal behaviour is converted into a Langmuir binding isotherm at high concentrations of the water oxidase (see text).

Can the observed concentration dependence be explained under the assumption of an irreversible reaction of hydroxylamine in the dark? The induction up to about 3 μM can. If there were hydroxylamine-trapping groups with a capacity of say 3 μM and with a very high affinity, there was indeed no effect expected before saturation of these groups. Beyond this, however, an irreversible reaction would proceed unless all water oxidase centres were saturated. At low concentrations this may go on slowly but it proceeds to completion. Since this consumed only some multiple of 40 nM, the concentration of the water oxidase in our samples, we expected a concentration dependence after the induction which was steeper than the observed one (see Fig. 4*a*). Note that the data shown in Fig. 4*a* were obtained after the effect was stationary. This and the above described washing experiment showed that the reaction was reversible.

What if the reversibility was indirect? Imagine an irreversible reaction of the centre with hydroxylamine, e.g. a two-electron reduction from state S_1 to S_{-1}, followed by a slower reoxidation of S_{-1} back to S_1 caused by some other agent, for example dioxygen. Then the effect *vs.* concentration data would not reflect a binding isotherm but rather the stationary level of the intermediate, S_{-1}, of a consecutive reaction. No such oxidation reaction is known so far, the only known spontaneous oxidation of the water oxidase is from state S_0 to S_1 caused by the intrinsic electron acceptor D^+ and proceeding in 20 min [27], which is too slow to be relevant in our context (see Fig. 3).

Based on the foregoing considerations we fitted the effect *vs.* concentration data for hydroxylamine by an isotherm for an enzyme with several co-operative binding sites. For the sake of simplicity we chose the Pauling–Adair model of sequential interaction with only three fit parameters, namely n, the number of binding sites, K, the dissociation constant of the first ligand, and a, the interaction factor, the same factor valid for all occupation states. This is illustrated in Fig. 4*b*. As common, these three parameters are sufficient to provide considerable ambiguity. Three fit curves are plotted in Fig. 4*a*. For two of these curves it is assumed that the effect is the same independent of whether 1, 2, 3, or more sites are occupied. (Fig. 4*a*, dashed and dotted lines.) They resulted in the following parameter sets (n, K, a): 4, 97 μM, 0.2; and 3, 152 μM, 0.05 [25]. For the third fit we assume that

the minimum requirement is an occupation by 2 molecules of hydroxylamine, but three and four giving the same effect as two (Fig. 4*a*, solid line). The fit parameters are 4, 44 μM, and 0.325. We do not wish to overstress the better quality of the last fit. The common features of all fits are as follows:

The water oxidase in its oxidation state S_1 contains 3–4 sites for the reversible binding of hydroxylamine. These are cooperatively populated (Hill coefficient 2.4). The effect of bound hydroxylamine which is observed after excitation of PS II with one flash of light is the same once the occupation number exceeds a minimum value (1 or 2 mol).

Which are the sites of hydroxylamine binding? According to Sandusky and Yocum [28] and Beck and Brudvig [29] there are at least two classes of binding sites for primary and secondary amines. The first class is possibly associated with the manganese centre proper and the second one with the sites of chloride binding in the vicinity of the centre. We examined the possibility that hydroxylamine is bound to the latter class. The effect *vs.* concentration behaviour was redetermined under variation of the chloride concentration. As documented by the open triangles in Fig. 4*a* there was no influence of chloride on the binding curve in the concentration range between 100 μM and 30 mM. If hydroxylamine was replacing chloride at its binding site an effect of chloride was to be expected.

For the binding of hydroxylamine to the manganese centre we have previously proposed a model where two intrinsic bridges, e.g. μ-oxo bridges between a binuclear manganese centre, are replaced either by coordination of 4 molecules of hydroxylamine or, alternatively, by 2 molecules of hydrazine as bridging ligand [26]. Only amino groups interact with manganese. This model most naturally incorporates the different Hill coefficients, 2.43 indicative of three if not four sites for hydroxylamine, and 1.48 indicative of two (or more) sites for hydrazine. It makes falsifiable predictions on Mn–Mn distances, 4.2 Å with hydrazine instead of 2.7 Å in controls and about 5.7 Å with hydroxylamine [26]. The enlarged distances are based on the known crystal structure of manganese–hydroxylamine complexes [30].

The larger Mn–Mn distances have not been detected in EXAFS studies with hydroxylamine treated samples ([31, 32] and Sauer *et al.*, this issue). This may indicate that there is indeed no enlargement of distances. However, a word of caution may be worthwhile. Very different sample concentrations are used in optical spectroscopy (our approach) and in EPR or in EXAFS. We work with thylakoid suspensions containing only 20 μM chlorophyll, the concentration of water oxidases is about 40 nM. Out of the 10 μM of hydroxylamine required for the expression of the effects only a very small portion will be bound. So the total concentration of hydroxylamine is very close to the concentration of free hydroxylamine. This is totally different in concentrated samples of approximately 10 mM-Chl and 4 μM water oxidases as used in EPR and EXAFS studies. Using the fit parameters based on our experiments with diluted samples (see Fig. 4) we calculated the concentration dependence of the effect of hydroxylamine in samples with micromolar concentrations of the water oxidase. In that calculation we assumed that there were no binding sites for hydroxylamine other than the cooperative ones (Fig. 4*b*). It is intuitively apparent that for a concentrated sample the concentration of free hydroxylamine over the total concentration will be flattened the most in the range where the cooperative binding curve is steepest. This 'buffering' also flattens the effect *vs.* concentration curves as documented by the simulated curves in Fig. 5. This particular simulation was carried out for the parameter set $\{(n;\ K;\ a) = (4;\ 97\ \mu\text{M};\ 0.2)\}$ and under consideration of the relation between free and total hydroxylamine. It is important to note that the effect *vs.* concentration dependence approaches the one of a simple first-order binding isotherm in concentrated samples, although the effect *vs.* free concentration behaviour, is highly cooperative and sigmoidal. The published EXAFS work was carried out with samples containing about 14 μM of water oxidases. The authors observed an influence of 40 μM hydroxylamine on about 65% of the centres as measured by the disappearance of the multiline EPR signal [32]. This agrees with our prediction (see Fig. 5, "14 μM"). Fig. 4(*c* and *d*) shows the frequency of occupation numbers, 0, 1, 2, 3, 4. With the percentage of modified centres being 65% (i.e. $1+2+3+4$ or $2+3+4 = 65\%$ depending on the respective parameter set) we found all occupation states, broadly speaking, about equally probable. This implied that the EXAFS work might have been carried out with an ensemble of water oxidases which was strongly heterogeneous with respect to the ligand state of the water oxidase.

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