Biochimica et Biophysica Acta 810 (1985) 225–234
Elsevier

BBA 41871

Studies on the interaction between hydroxylamine and hydrazine as substrate analogues and the water-oxidizing enzyme system in isolated spinach chloroplasts

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(Received May 30th, 1985)

Key words: Photosystem II; Hydroxylamine; Hydrazine; Water splitting; Oxygen evolution; (Spinach chloroplast)

The functional interaction between the photosynthetic water-oxidizing enzyme system and the substrate analogues hydroxylamine and hydrazine has been analyzed in isolated class II chloroplasts by measuring the effect of these species on the characteristic oscillation pattern of oxygen yield induced by a flash train. The following was found. (1) At concentrations where both substances cause the pronounced two-flash phase shift (Bouges, B. (1971) Biochim. Biophys. Acta 234, 103–112) the dark equilibration is rather slow with half-times of approx. 1 min. (2) The numerical evaluation of the oscillation patterns reveals quantitative differences between hydroxylamine and hydrazine. The interaction with hydroxylamine is complex. It involves one- and two-electron processes as well as fast reaction steps during the flash sequence. The fast reactions take place only with redox states S_2 and S_3 of the water-oxidizing enzyme. Furthermore, the redox turnover in the presence of hydroxylamine leads to an S_1-state that differs markedly in its susceptibility to hydroxylamine from that of S_1 in control chloroplasts. (3) Below a threshold concentration which varies for different preparations the hydrazine effect can be quantitatively described by the assumption that after dark equilibration the agent becomes consumed irreversibly via a reaction with two oxidizing redox equivalents produced by PS II. This process is accomplished during the first two flashes. No further interaction occurs during the flash sequence, so that besides the two-flash phase shift the water-oxidizing enzyme system reveals the normal oxygen-evolution pattern. (4) Based on the analysis of the concentration dependence hydrazine is inferred to interact with the catalytic center of the water-oxidizing enzyme system via a cooperative mechanism including two binding sites. The data are discussed in terms of the kinetics of the dark interaction and its possible rate limitation. Mechanistic aspects (ligand–ligand exchange at the functional manganese cluster and transport step) are considered. Furthermore, possible mechanisms for the redox reaction of hydrazine at the catalytic site are briefly discussed.

Introduction

The kinetics of photosynthetic water oxidation to dioxygen are widely resolved, but many essential mechanistic aspects, such as the chemical nature of the intermediary redox states, the structure of the catalytic manganese cluster and the structure as well as the functional role of the apoenzyme including regulatory subunits still remain to be clarified (for a recent review, see Ref. 1). Among different ways to attack these problems, a well-defined modification of the reaction pattern by selectively acting agents appear to be one suitable

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technique. Two different classes of specifically interacting substances are of special interest: (a) ADRY-agents that drastically modify the lifetimes of the redox states S_2 and S_3 in the water-oxidizing enzyme system [2,3] and (b) substances acting as water analogues that compete effectively at the catalytic center with the natural substrate H_2O for oxidizing redox equivalents. Hydroxylamine (NH_2OH) at low concentrations (in the micromolar range) was found to shift the characteristic oscillation pattern of flash-induced oxygen evolution in dark adapted chloroplasts by a number of two [4], i.e., in the presence of 10–50 μM NH_2OH the maxima of the oxygen yield are observed in the 5th, 9th and 13th flash instead of in the 3rd, 7th and 11th flash as in normal chloroplasts. Similar effects were observed for hydrazine [5] and H_2O_2 [6]. At higher concentrations the water-oxidizing enzyme systems becomes irreversibly destroyed [7]. This effect, however, will not be analyzed explicitly in this study.

Mass spectroscopic studies led to the conclusion that N_2 is the only stable volatile product of NH_2OH and hydrazine (N_2H_4) oxidation [8,9]. Based on these experiments and on sterical considerations Radmer and Ollinger came to the conclusion that after reduction of S_1 to S_0 by NH_2OH two water molecules at the functional binuclear manganese center are replaced by one NH_2OH-molecule [9–11]. After flash excitation, the S_0-NH_2OH complex decomposes and the water-oxidizing enzyme systems starts the normal 4-step univalent redox cycle for water oxidation from S_0. Recently, the mechanistic studies about the interaction of hydroxylamine with the water-oxydizing enzyme system were extended by analyzing the proton release pattern. It was shown that in the presence of NH_2OH two protons are released due to excitation with the first flash. The flash induced proton release pattern in dark adapted NH_2OH-treated chloroplasts was inferred to be inconsistent with the mechanism proposed by Radmer and Ollinger [12].

An interesting hypothetical mechanism for the action of NH_2OH in the water-oxidizing enzyme system was discussed by Förster [13]. The proposed scheme implies the replacement of two water molecules by two NH_2OH-molecules in state S_1 of the binuclear catalytic manganese center. Oxidation of this complex with a single turnover flash leads to S_0 formation under release of two H^+ and one N_2-molecule [13]. However, as emphasized by the author this hypothetical mechanism remains to be confirmed experimentally.

The interference of comparatively low concentrations of the water analogs NH_2OH and N_2H_4 with the catalytic manganese center involves binding and ligand exchange reactions. The present study is an attempt to characterize these reactions.

Materials and Methods

Isolation of thylakoids from spinach (*Spinacea oleracea*) was performed as described in Ref. 14, except for the centrifugation procedure. Thylakoids were centrifuged at 5000 × *g* for 30 s in both steps. The number of intact water-oxidizing enzyme systems was found to be one per approx. 500 chlorophylls, as determined by conventional flash induced oxygen yield measurements [15].

Oscillation patterns of oxygen yield in dark-adapted thylakoids were detected with an unmodulated Joliot-type oxygen electrode [16]. The thylakoid suspension (chlorophyll concentration, approx. 1 mM) on the Pt electrode was separated by a single dialysis membrane from the upper buffer compartment containing the Ag/AgCl-reference electrode. Thylakoids were isolated, stored on ice and transferred to the electrode in darkness. After 5 min for equilbration on the electrode, the thylakoids were illuminated with a series of saturating flashes (10 μs half-width) using a Stroboslave 1539-A (General Radio). The signals were digitally recorded with a Nicolet Explorer III storage oscilloscope. The amplitudes were used to indicate the amount of oxygen produced. For normalization of the flash yields (all data are normalized unless otherwise mentioned) as well as for calculations on the relative S_i-state concentrations according to the Kok model [17] the procedure extensively described in Ref. 3 was used. The thylakoid suspension was mixed with hydoxylamine or hydrazine directly before being used in the experiment. The buffer solution contained: 10 mM NaCl/5 mM $MgCl_2$/0.3 M sorbitol/50 mM tricine-NaOH (pH = 7.6).

Results

A typical oscillation pattern of the flash-induced oxygen yield in control samples and in class II-chloroplasts preincubated in the dark with 200 μM and 300 μM hydrazine (N_2H_4) is depicted in Fig. 1. At 300 μM, the typical N_2H_4-induced shift of the oxygen-yield maxima by a number of two flashes is observed. A qualitatively similar effect is found for NH_2OH in agreement with previous reports [4,10]. For a quantitative description of the experimental data informations are required about the interference mechanism of water analogues with the catalytic manganese center for water oxidation. The binding and ligand exchange mechanism can be analyzed without detailed knowledge about the redox chemistry at the catalytic site, provided that the following conditions are satisfied. (a) The binding and exchange rates of the water analogues are slow enough, so that during the flash sequence neither rebinding nor redistribution of these species between different water-oxidizing enzyme systems takes place. (b) The equilibrium state is achieved during the dark preincubation. (c) Interaction between water analogues and the water-oxidizing enzyme system causes only two effects, that are concentration dependent – below a threshold number of bound molecules, the S-state vector is modified in a way that causes a shift by two flashes of the oxygen-yield pattern during the measurement, while above this threshold, water-oxidizing enzyme systems are irreversibly destroyed, the percentage of these inactivated systems does not change during the flash sequence.

Accordingly, the samples preincubated with water analogue consist of a population of three different types of water-oxidizing enzyme systems: (1) unmodified; (2) modified in terms of a two flash-shifted oscillation pattern, and (3) destroyed systems. If the oxygen-yield values of the flash sequence are appropriately normalized (as outlined in Ref. 3), the effect of irreversible destruction is eliminated and the normalized oxygen yield pattern, $Y_n(c)$, can be described by the relation:

$$Y_n(c) = p(c)\ Y_n(\text{mod}) + [1 - p(c)]\ Yn(\text{unmod}) \qquad (1)$$

where $p(c)$ is the concentration-dependent probability for a modification of the water-oxidizing enzyme system into a state that gives rise to a two-flash-shifted oscillation pattern, Y_n(unmod) and Y_n(mod) are the oscillation patterns of the control and completely modified samples, respectively.

A numerical fit of the experimental data in Fig. 1 reveals that a satisfying description can be achieved for N_2H_4 at the particular concentrations of 200 μM and 300 μM (a more detailed analysis is given at the end of this chapter). In order to make sure that the data of Fig. 1 really reflect the achievement of an equilibrium state for N_2H_4-binding, the effect of dark incubation time on the modification rate of functional centers as well as on the disappearance of this effect was measured. A rough quantitative measure for the binding rate is the dependence of the normalized oxygen yield in the third flash, Y_3, on the dark incubation time. The data in Fig. 2 show that at constant NH_2OH- and N_2H_4-concentration Y_3 continuously declines with increasing dark incubation time. A numerical analysis of the data reveals that in both cases a monoexponential decay takes place with half-times of 1.2 and 1.1 min, respectively. As the concentrations of both species exceed the number of water-oxidizing enzyme systems by more than one order of magnitude, a pseudo-first-order binding reaction can be ex-

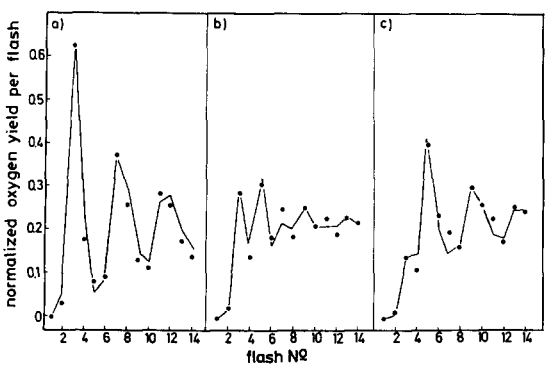


Fig. 1. Normalized oxygen yields as a function of flash number in dark-adapted spinach chloroplasts. (a) Control; (b) 200 μM N_2H_4; (c) 300 μM N_2H_4. Theoretical curves were calculated according to the Kok-scheme with $[S_0] = 0$; $[S_1] = 1.0$; misses, $\alpha = 0.12$; double hits, $\beta = 0.03$. The modification parameter $p(c)$ was 0.54 for experiment (b) and 0.78 for experiment (c). Experimental data are represented by dots. For further details see text.

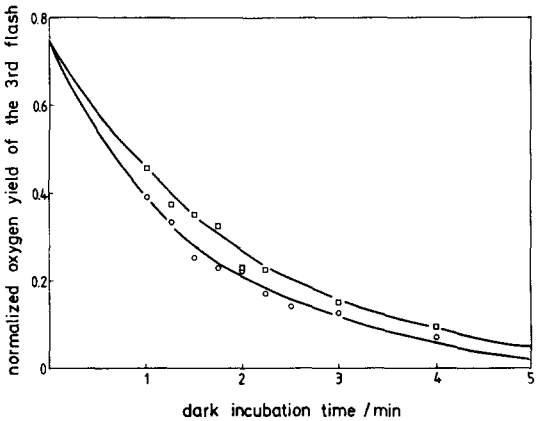


Fig. 2. Normalized oxygen yield of the third flash, Y_3, as a function of dark incubation time in the presence of 50 μM NH_2OH (○) or 250 μM N_2H_4 (□).

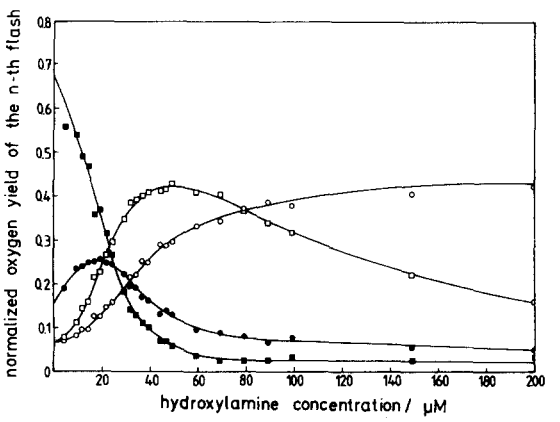


Fig. 3. Normalized oxygen yields as a function of NH_2OH-concentration in dark equilibrated (15 min) spinach chloroplasts. ■, Y_3, ●, Y_4, □, Y_5, ○, Y_6.

pected. Taking into account the different concentrations for NH_2OH and N_2H_4, the data also suggest that the concentration-independent rate constant, k_{bind}, for NH_2OH appears to be larger than that for N_2H_4. Regardless of these quantitative differences the binding rate is comparatively slow for both substances. This indicates that there probably exists either a high transport barrier and/or a slow ligand exchange (see Discussion).

In order to test whether or not the interaction between N_2H_4 and the water-oxidizing enzyme in the dark is a reversible binding process, the N_2H_4-release was measured. After dark incubation (5 min) with N_2H_4 class II chloroplasts were centrifuged and rapidly resuspended in N_2H_4-free buffer solution. At definite dark times after resuspension these chloroplasts were transferred to the Joliot-type electrode and oxygen-yield pattern was measured. An increase of the ratio Y_3/Y_5 was observed with increasing dark time, which provides a measure for the N_2H_4-release in the dark. Based on these measurements, the N_2H_4-release was estimated to occur with min-kinetics. These data reveal that the interaction between N_2H_4 and the water-oxidizing enzyme in the dark is predominantly a reversible process.

In order to analyze whether the effects of NH_2OH and of N_2H_4 can be satisfactorily described by Eqn. 1 in a wide concentration range, a more detailed analysis was performed. Fig. 3 shows the normalized oxygen yield of the 3rd, 4th, 5th and 6th flash of the sequence as a function of NH_2OH-concentration. In order to eliminate effects due to the limited binding rate at low NH_2OH-concentrations, class II chloroplasts were incubated in the dark with NH_2OH for 15 min. The data of Fig. 3 reveal that Y_3 steeply declines with NH_2OH concentration, attaining a constant small value at approx. 70 μM NH_2OH. A further analysis on the basis of the above-mentioned conditions included in Eqn. 1 predicts that at increasing NH_2OH-concentrations Y_5 and Y_6 should continuously rise up to a constant value, whereas only slight changes are expected for Y_4. The experimental data show that Y_5 reaches a maximum value at almost the same NH_2OH-concentration, where Y_3 attains its minimum value. However, at higher NH_2OH concentrations Y_5 starts to decline. This deviation from the expected pattern indicates that at higher NH_2OH-concentrations Eqn. 1 fails to describe the experimental data. Therefore, one of the above-mentioned conditions (a)–(c) is not valid. A likely explanation for the phenomenon of a Y_5-decline with increasing NH_2OH-concentrations is the possibility that NH_2OH additionally interacts during the flash sequence with redox equivalents stored in the water-oxidizing enzyme system. In this case in addition to the two-flash phase shift of the oxygen yield pattern caused by dark equilibration with NH_2OH some of the water-oxidizing enzyme systems would undergo a further NH_2OH-induced modification of the S-

state vector by the loss of oxidizing redox equivalents during the flash sequence. In order to characterize the kinetics of this type of NH_2OH-interaction, the ratio Y_5/Y_6 as a function of the dark time between the flashes was determined as a qualitative measure. The experimental data obtained at a NH_2OH-concentration of 50 μM are depicted in Fig. 4. The ratio Y_5/Y_6 monoexponentially declines with an apparent rate constant, k_{app} of 1.3 s^{-1}. The apparent half time of this decay knetics ($t_{1/2}$ = 530 ms) is faster than the NH_2OH-binding kinetics in the dark by more than one order of magnitude. Even though the ratio Y_5/Y_6 reflects only qualitatively the kinetics of NH_2OH-interaction during the flash sequence, the data of Fig. 4 reveal that this process appears to be markedly faster than the NH_2OH binding to the water-oxidizing enzyme in dark adapted chloroplasts.

In order to explain the different interaction kinetics during dark incubation and the flash sequence, respectively, two basically distinct phenomena have to be taken into consideration: (a) light-induced conformational changes that could accelerate the rate-limiting step of the overall reaction of dark equilibration; or (b) both processes (dark vs. light) are unrelated phenomena that are caused by different underlying mechanisms.

These alternative interpretations can be experimentally tested by analyzing whether or not the interaction between NH_2OH and the water-oxidizing enzyme during the flash sequence depends on the S_i-states. Accordingly, the following experiments were performed: after sufficiently long dark incubation with NH_2OH chloroplasts were illuminated with a flash train at a constant dark time between the flashes of 300 ms, except of one flash pair in the sequence, that is spaced by a varying dark time $\Delta t_{n,n+1}$ between flashes number n and $n+1$. If one accepts that a two-flash phase shift occurs due to NH_2OH dark incubation [4,9–13], then after one flash the water-oxidizing enzyme systems of the sample mainly attain the state S_0. Likewise, after two, three and four flashes the redox states S_1, S_2 and S_3, respectively, dominate. Therefore the effect of a variation of the dark time between the first and second flash, $\Delta t_{1,2}$, on the normalized oxygen yield pattern should reflect the interaction kinetics between S_0 and NH_2OH. Analogously the effects of $\Delta t_{2,3}$, $\Delta t_{3,4}$ and $\Delta t_{4,5}$ would provide information about the corresponding reactions between NH_2OH and S_1, S_2 and S_3, respectively. The experimental data obtained are depicted in Fig. 5. At a first glance the results readily show drastic differences in the reaction behavior of S_0 and S_1 vs. S_2 and S_3. The comparatively fast interaction between NH_2OH and the water-oxidizing enzyme system during the flash sequence only occurs in states S_2 and S_3. These findings show that a simple acceleration of the rate-limiting step for interaction in the dark

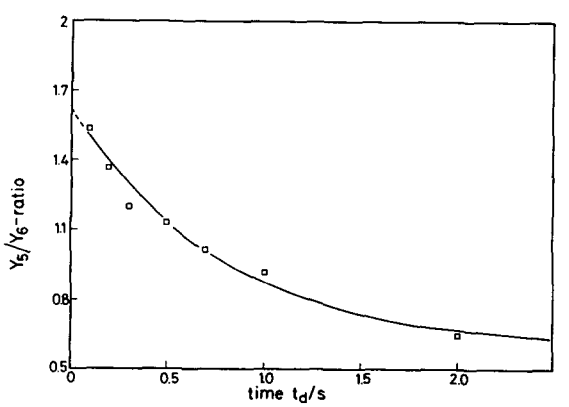


Fig. 4. Y_5/Y_6-ratio as a function of dark time between the flashes in spinach chloroplasts dark equilibrated for 15 min with 50 μM NH_2OH.

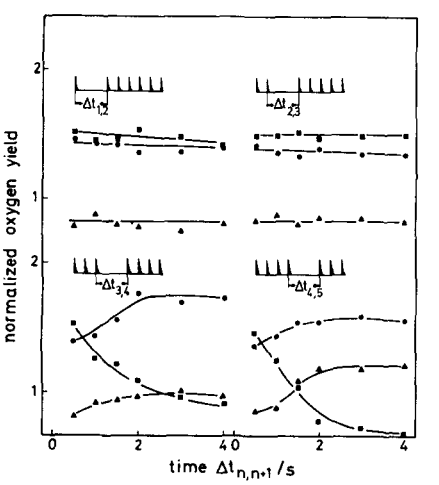


Fig. 5. Normalized oxygen yields of the 5th, 6th and 7th flash as a function of the dark times $\Delta t_{1,2}$, $\Delta t_{2,3}$, $\Delta t_{3,4}$ and $\Delta t_{4,5}$, respectively, in spinach chloroplasts dark equilibrated for 15 min with 50 μM NH_2OH. ■, Y_5; ●, Y_6; ▲, Y_7.

(alternative a) can be excluded as the dominating mechanism for the fast interaction during the flash sequence, because dark equilibration takes place between NH_2OH and the water-oxidizing enzyme system in redox state S_1 and S_0, whereas the fast process occurs only in state S_2 and S_3.

Now the question arises about the mechanism that is responsible for the fast reaction with S_2 and S_3. An important mechanistic aspect is the problem of the redox stoichiometry, i.e., whether mono- or bivalent redox reactions dominate. This problem can be attacked by the following qualitative considerations. If one assumes that in NH_2OH-incubated chloroplasts after three flashes the state S_2 is mainly populated, one would expect in case of a univalent S_2 decay to S_1 that the normalized oxygen yield Y_5 declines with increasing $\Delta t_{3,4}$, whereas Y_6 increases and Y_7 is only slightly affected. On the other hand if S_2 would decay via a bivalent process to S_0, then Y_5 and Y_6 should decrease with increasing $\Delta t_{3,4}$, whereas Y_7 should correspondingly be enhanced. An inspection of the experimental data shows that a univalent redox mechanism appears to be more likely. An analogous consideration for the anticipated pattern of Y_5, Y_6 and Y_7 as a function of $\Delta t_{4,5}$ and a comparison with the experimental data also favors a univalent reaction mechanism ($S_2 \rightarrow S_1$, $S_3 \rightarrow S_2 \rightarrow S_1$). Accordingly, one can infer that the fast interaction between NH_2OH and S_2 and S_3 is dominated by univalent redox reactions. This conclusion is supported by the fact that Y_4 increases at low NH_2OH-concentrations (see Fig. 3).

An increase of Y_4 has already been observed in normal chloroplasts if the dark time between the flashes increases [18]. This effect that phenomenologically enhances the 'apparent' S_0 population was explained by the existence of a one-electron donor component in approx. 20% of all systems II that turns over only once during the flash sequence and reduces S_2 (to S_1) and/or S_3 (to S_2). In the presence of NH_2OH an analogous effect could be achieved by NH_2OH itself or by NH_2OH induced stimulation of the above-mentioned internal pathway. If one assumes that donor D (giving rise in its oxidized form to signal II_s) is responsible for the fast S_2 (and S_3) decay in the control [18], NH_2OH could induce $D^{ox}(II_s)$ reduction. This idea remains to be tested experimentally. Regardless of these mechanistic details, the results of Figs. 3–5 show that NH_2OH interacts with the water-oxidizing enzyme system in a more complex way than predicted by Eqn. 1, which includes the above-mentioned assumption (a)–(c).

Fig. 5 reveals a fast NH_2OH-induced S_2- and S_3-decay. Accordingly, the question arises, whether or not the states S_0 and S_1, that are assumed to be formed by excitation with one and two flashes, respectively, in NH_2OH- and/or N_2H_4-incubated chloroplasts, also differ from the states S_0 and S_1 in control chloroplasts. In order to test this problem chloroplasts were equilibrated with either 50 μM NH_2OH or 300 μM N_2H_4 in the dark, illuminated by one or two preflashes, and subsequently kept in dark for varying times before measuring the flash-induced oxygen yield pattern.

The flash yields of the 3rd and 4th flash of the sequence (Y_3, Y_4) were used as a qualitative measure of the S_1 and S_0 population that exists just before illumination with the flash train. The data depicted in Fig. 6 exhibit two rather interesting phenomena. (i) The normalized Y_3 and Y_4 values remain almost constant within a time domain of 20 min, if preillumination was performed with two flashes. This indicates that the presumed S_1-state occurring after a two-flash preillumination of thylakoids in the presence of NH_2OH (N_2H_4) reacts at least 20-times slower with NH_2OH (N_2H_4) than S_1 in control thylakoids. (ii) The Y_3

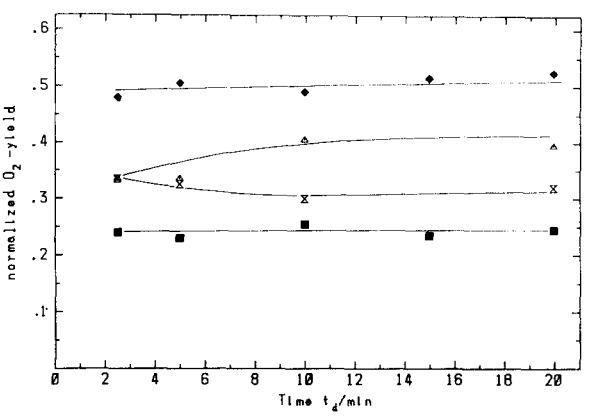


Fig. 6. Normalized oxygen yield of the third and fourth flash, Y_3 and Y_4, as a function of N_2H_4 (300 μM)-incubation time in the dark after illumination with one or two preflashes. ◆, $1Y_3$ (two preflashes), ■, Y_4 (two preflashes), △, Y_3 (one preflash), X, Y_4 (one preflash). Similar results were obtained in the presence of 50 μM NH_2OH.

values after one-flash preillumination slightly increases with increasing dark time, whereas the Y_4 values exhibit a small decrease. The changes of Y_3 and Y_4 after a one-flash preillumination can be interpreted as a slow transformation of S_0 into S_1 that resembles that in control chloroplasts without NH_2OH [18].

The results of Fig. 6 led to the conclusion that the S_1-state produced in NH_2OH-incubated and in NH_2H_4-incubated chloroplasts, and referred to as $S_1(NH_2OH)$ and $S_1(N_2H_4)$, respectively, differ markedly with respect to the NH_2OH (N_2H_4)-susceptibility of S_1 in normal chloroplasts. It has recently been shown [19] that the water-oxidizing enzyme system undergoes a very slow transition into a resting state. This transformation is very likely coupled with conformational changes. It therefore appears to be very attractive to speculate about the possibility that the turnover of NH_2OH (N_2H_4) close to the manganese-containing functional catalytic site could also cause conformational changes that modify the microenvironment giving rise to a different susceptibility to NH_2OH (N_2H_4) without affecting the S_i-state redox transitions leading to oxygen formation. Experiments are in progress to attack this problem.

The data show that the binding and ligand–ligand exchange interaction between NH_2OH and the water-oxidizing enzyme system is a rather complex phenomenon. Especially the fast S_2- and S_3-decay in the presence of NH_2OH has to be considered in order to avoid serious misinterpretations of experimental data.

Another water analogue that has been analyzed for its interaction with the water oxidizing enzyme is N_2H_4. As already shown in Fig. 1, the effect of this component on the flash-induced oscillation pattern of the oxygen yield can be satisfactorily described by Eqn. 1 at particular concentrations of 200 μM and 300 μM. In order to show whether or not this relation also describes the N_2H_4-induced effect over a wider concentration range, the normalized values of Y_3, Y_4, Y_5 and Y_6 were measured as a function of N_2H_4-concentration. The experimental data obtained are depicted in Fig. 7. In contrast to the situation with NH_2OH, the results of Fig. 7 exhibit a pattern in the concentration range of 50–300 μM N_2H_4 that is consistent with the assumptions implied in Eqn. 1. It is especially

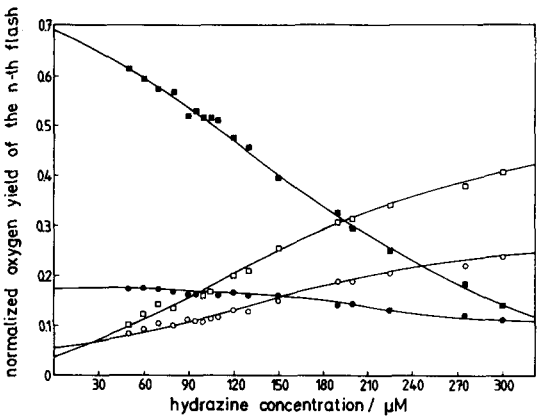


Fig. 7. Normalized oxygen yields as a function of N_2H_4-concentration in dark equilibrated (15 min) spinach chloroplasts. ■, Y_3; ●, Y_4; □, Y_5; ○, Y_6.

interesting to note that in the presence of N_2H_4 the normalized oxygen yield Y_4 does not increase with increasing N_2H_4 concentration and remains almost constant as predicted by Eqn. 1. Furthermore, a comparison of Fig. 3 and 7 shows that NH_2OH exerts an approx. 5-fold stronger effect compared with N_2H_4, on the basis of the molar concentration ratios. It should be mentioned that the N_2H_4-concentration effect varies slightly for different preparations.

The finding that at sufficiently low concentrations Eqn. 1 is appropriate to describe the N_2H_4-effect over a sufficiently wide concentration range offers the possibility to analyze the thermodynamics of N_2H_4-binding to the water-oxidizing enzyme system. The parameter, $p(c)$ in Eqn. 1 reflects the degree of N_2H_4-occupancy of the catalytic center within the water-oxidizing enzyme by N_2H_4. Accordingly, parameter $p(c)$ as a function of the N_2H_4-concentration is expected to provide information about the binding mechanism. Experiments were performed at different N_2H_4-concentrations and the parameter $p(c)$ was determined by fitting the experimental data with Eqn. 1. The results obtained are depicted in Fig. 8. The most striking phenomenon of the N_2H_4-concentration dependence of $p(c)$ is the finding of a sigmoidal binding curve. This phenomenon very likely indicates some cooperativity of different binding sites. It has been previously discussed that the catalytic center of the water-oxidizing enzyme

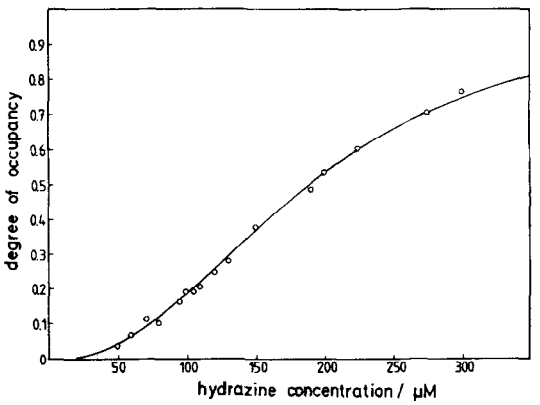
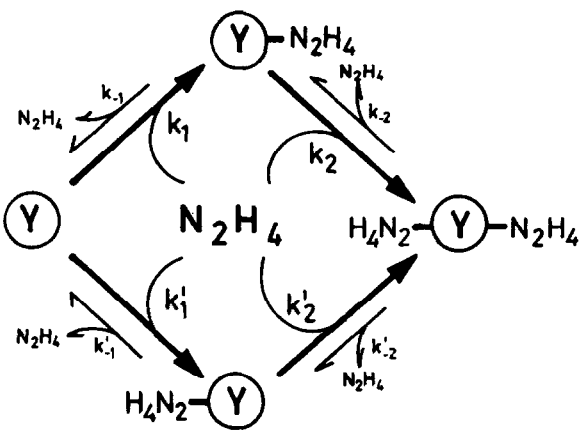


Fig. 8. Interaction parameter $p(c)$ as a function of N_2H_4-concentration in dark equilibrated (15 min) spinach chloroplasts. Parameter $p(c)$ was calculated by the use of Eqn. 1 from the experimental data of Fig. 6 (for details see text). The fitting curve was obtained by using Eqn. 2.

system probably contains a binuclear manganese cluster that stabilizes redox intermediates of photosynthetic water oxidation [20–22]. Accordingly, it appears reasonable to assume that each manganese provides a binding site for N_2H_4. In this case the binding mechanism can be described in the following way:



Scheme I.

where Y symbolizes the water-oxidizing enzyme system and subscripts 1 and 2 indicate the two binding sites for N_2H_4. The ratios of the rate constants k_i/k_{-i} denote the equilibrium constants for the different binding steps, i.e., $k_i/_{-i} = K_i$. If one assumes that in the proposed model (Scheme I the two binding sites are indistinguishable, then for the sake of symmetry the following relations hold: $K_1 = K_1'$ and $K_2 = K_2'$. Furthermore, only molar ratios of the reactants will be used. Then, on the basis of two binding sites the generalized Adair equation (Ref. 23) leads to Eqn. 2:

$$p(N_2H_4) = \frac{K_1[N_2H_4] + K_1K_2[N_2H_4]^2}{1 + 2K_1[N_2H_4] + K_1K_2[N_2H_4]^2} \quad (2)$$

where $[N_2H_4]$ represents the normalized N_2H_4-concentration. A fitting of the data in Fig. 8 by a nonlinear parametric method leads to the following values: $K_1 = 5{,}2 \cdot 10^2\ M^{-1}$ and $K_2 = 5.6 \cdot 10^4\ M^{-1}$ a.u. (arbitrary units). Within the framework of this model the N_2H_4-binding to the water-oxidizing enzyme system can be described by a cooperative mechanism including two binding sites. The ratio of the equilibrium constants K_2/K_1 indicate that after attachment of one hydrazine molecule the binding of the second molecule occurs with a 100-fold higher affinity. From this value the free interaction energy can be calculated. According to Wyman's relation (Ref. 24) one obtains:

$$\Delta G_I = -RT \ln \frac{K_2}{K_1} = -11.5\ \text{kJ/mol} \quad (3)$$

This value corresponds to a weak interaction in the order of a moderate hydrogen bond.

Discussion

The data in this study show that the achievement of the binding equilibrium between the substrate analogues NH_2OH and N_2H_4 and the water-oxidizing enzyme system in the dark is a comparatively slow process. If one assumes that the modification of the characteristic oscillation pattern of the oxygen yield by NH_2OH and N_2H_4 requires the binding of these agents to the catalytic manganese center, and if one takes into account that the redox steps are not rate limiting (the phase shift takes place at a dark time of 300 ms between the flashes), then two factors could be responsible for the slow overall reaction in the minute range: (a) transport of the species to the catalytic center and/or (b) ligand-ligand exchange

at the functional manganese group. Using the data of Fig. 2 a rough calculation indicates that the binuclear rate constant for a process of the type $Mn \cdot L + NH_2OH(N_2H_4) \rightarrow Mn \cdot NH_2OH(N_2H_4) + L$ is of the order of approx. 100 $M^{-1} \cdot s^1$. If one assumes that the ligand exchange is fast, then the rate of the overall reaction would be limited by a significant retardation of the transport process from the outer bulk phase to the catalytic site. As this group is located within an intrinsic polypeptide close to the thylakoid lumen one could speculate about the transmembrane equilibration of NH_2OH and N_2H_4 as rate-limiting step. However, preliminary data indicate, that the equilibration rate with the catalytic center in inside-out thylakoids is very similar to that in normal thylakoids (Diedrich-Glaubitz, R., Gräber, P. and Renger, G., unpublished results). Accordingly, if the transport process is really rate limiting, it appears to be more likely that surface-exposed polypeptides, especially the 33 kDa unit covering up the catalytic manganese cluster (for a review, see Ref. 1), play an essential role as transport barrier. Ligand–ligand exchange rates at metal centers can vary over many orders of magnitude, depending on the nature of the ligand, the nature of the metal ion and its valence state (for a review, see Ref. 25). Therefore, in principle the rate of dark equilibration of the water-oxidizing enzyme system with NH_2OH and N_2H_4 could be limited by the ligand–ligand exchange reaction. No detailed informations are available for the structure of the ligand shell at the functional (binuclear) manganese center and even the valence state is not unambiguously clarified. However, if one accepts that the Mn(III) state plays an essential role (for a review, see Ref. 1) a comparison with in vitro data for the interaction kinetics between $Mn(III)_{aq}$ and NH_2OH or N_2H_4 [26] do not support the above-mentioned idea. Because of the complexity of the chemical environment it has to be emphasized that our present data do not permit to draw any further conclusions about the mechanistic details that are responsible for the rate limitation of the overall reaction. Likewise, the much faster interaction of NH_2OH with S_2 and S_3 remains to be clarified in respect to the underlying reaction mechanism.

Generally, two different mechanisms could be considered: (a) direct interaction with the functional manganese center in states S_2 and S_3, or (b) indirect effect via dissipation of redox equivalents at the PS II donor side that eventually leads to S_2- and S_3-decay via univalent redox reactions. This type of mechanism could resemble other types of reactions that are known to dissipate S_2 and S_3 (for a recent review, see Ref. 27). The data of this paper do not permit to give an unambiguous decision. Experiments are in progress to clarify this point. A remarkably simpler pattern is observed for the interaction between N_2H_4 at sufficiently low concentrations and the water-oxidizing enzyme system.

It was found that Eqn. 1 quantitatively describes the oscillation patterns within a concentration range of 50–300 μM N_2H_4. Accordingly parameter $p(c)$ could be determined as a function of the N_2H_4-concentration. The numerical fit of the data led to the conclusion that the interaction between the functional manganese group and the water-oxidizing enzyme system includes cooperative phenomena. As the data are obtained from measurements of effects on the oxygen yield any nonspecific binding effects that do not affect the functinal group are excluded. The most simple interpretation of the results is the assumption that two N_2H_4-molecules bind to the catalytic site. As this site very likely contains a binuclear manganese center, it appears reasonable to assume that each metal binds one N_2H_4-molecule via a process of ligand exchange. This is in line with the tendency of N_2H_4 to form mononuclear complexes [28]. In this respect it is interesting to note that the binding of the first N_2H_4-molecule markedly enhances the affinity for a second N_2H_4-molecule. This might suggest that conformational changes take place due to N_2H_4-H_2O-ligand exchange at the manganese center. Our data do not favour the idea of binuclear complexation of one N_2H_4 between two manganese centers. It should be mentioned that conclusions based only on the phenomenon of cooperativity cannot exclude the existence of a complex with two binuclearly bound N_2H_4-molecules as binding ligands between two manganeses. However, for sterical reasons this type of complex seems to be less likely than a mononuclear complexation. Despite of higher complexity in the interaction between NH_2OH and the functional

manganese center, a binuclear binding of only one molecule appears to be very unlikely. Therefore, our study supports the idea of Förster [13] that proposes the mononuclear binding of two NH_2OH-molecules. A last point which should be briefly discussed is the redox mechanism that causes the shift of the oxygen-yield pattern. The numerical analysis of the data depicted in Fig. 1 reveals, that the oscillation pattern in the presence of 300 μM N_2H_4 can be quantitatively described by the assumption that N_2H_4 feeds two electrons into the catalytic center of photosynthetic water oxidation without affecting the probability of misses and double hits. After N_2H_4-oxidation the normal turnover of the water-oxidizing enzyme system takes place. If one takes into account the experimental findings so far known, two possible mechanisms can be discussed: (a) formation of HN=NH and subsequent statistical disproportion ($2\ NH{=}NH \rightarrow N_2 + NH_2 - NH_2$) of the species generated in different water oxidizing enzyme systems; (b) N_2-formation at the catalytic center of each system by intrinsic oxidative disproportion of two complexed N_2H_4-molecules ($2\ \overset{2\oplus}{NH_2} - NH_2 \rightarrow 2\ NH_3 + N_2 + 2H^+$). The latter mechanism appears to be more attractive, because release and statistical disproportion of NH=NH should be affected by different side reactions. However, NH_3-formation has not been detected during N_2H_4-oxidation [9]. One might speculate that NH_3 could become internally trapped by protonation, so that it escaped the mass spectroscopic detection applied by Radmer and Ollinger.

For the time being, our data do not permit to draw any further conclusion about the redox mechanism. Possibly the combination with other techniques (e.g., time-resolved proton measurements) will provide further insight.

Acknowledgements

The authors would like to thank Dr. T. Wydrzynski for reading the manuscript. The financial support by the Deutsche Forschungsgemeinschaft (Re 354/8-1) is gratefully acknowledged.

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