Structural Changes in the Water-Oxidizing Complex Monitored via the pH Dependence of the Reduction Rate of Redox State S_1 by Hydrazine and Hydroxylamine in Isolated Spinach Thylakoids†

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ABSTRACT: A detailed kinetic analysis is presented for the pH dependence of the reduction of the water-oxidizing complex (WOC) in redox state S_1 by hydrophilic amines NH_2R (R = NH_2, OH) in suspensions of isolated thylakoids. Measurements of patterns of the oxygen yield induced by a train of single-turnover flashes and evaluation of the data within the framework of an extended Kok model [Messinger, J., Wacker, U., & Renger, G. (1991) *Biochemistry 30,* 7852−7862] led to the following results: (a) the rate constants $k_{S_1}(NH_2R)$ exhibit strikingly similar pH dependencies for NH_2OH and NH_2NH_2 with "titration waves" at pH 5.3−5.6; 6.2−6.5, and above a critical pH value of about 7.4; (b) the differences in the reaction mechanism between NH_2OH (1-electron reduction) and NH_2NH_2 (2-electron reduction) are almost pH-independent; (c) the ratio of the rate constants, $k_{S_1}(NH_2OH)/k_{S_1}(NH_2NH_2)$, decreases by a factor of about 9 within the range 5 < pH < 8.5. A detailed analysis reveals that these data cannot be consistently explained by the assumption that the unprotonated forms NH_2OH and NH_2NH_2 are the active species while the protonated cations $[NH_3OH]^+$ and $[N_2H_5]^+$ are nonreactive. A quantitative description is achieved by the additional postulate that pH-dependent structural changes take place in the WOC, thereby modulating the reactivity toward exogenous redox active amines of the type NH_2R. On the basis of the results of this study and a recent report [Messinger, J., & Renger, G. (1994) *Biochemistry 33,* 10896−10905], it is inferred that the WOC undergoes three specific structural changes, with characteristic pH values of 5.3−5.5, 6.2−6.5, and above 7.4.

Photosynthetic water oxidation to dioxygen takes place via a sequence of univalent redox steps within a manganese-containing functional unit referred to as the water-oxidizing complex (WOC).[1] This process is driven energetically by light-induced formation of the cation radical $P680^+$, with the redox active tyrosine Y_Z of polypeptide D1 acting as the intermediary electron carrier [for a review, see Renger (1992)]. Although the general functional organization scheme and the kinetic pattern of the elementary reactions are well resolved [for recent reviews, see Rutherford et al. (1992), Debus (1992), and Renger (1993)], a number of key mechanistic questions still remain to be answered [for a list, see Renger (1987, 1993)].

Among several fundamental problems, e.g., the nature of the oxidation steps in WOC (metal- or ligand-centred reactions), the structure of the manganese cluster and its coordination sphere, the template of substrate (water) binding and dioxygen formation, etc., structure−function relations in general are of central relevance for a satisfying understanding of the reaction mechanism. In principle, two types of structural effects have to be considered: (a) the dynamics of the nuclear geometry coupled with the individual redox transitions $S_i \rightarrow S_{i+1}$ in the WOC [see Kok et al. (1970)] and the formation of O_2 in the final oxidation step and (b) "static" conformational states of the WOC related to its overall functional activity. The former type of structural effect comprises several reactions, such as the binding of two water molecules to a suitable template, the possibility of hydrogen bond formation between substrate water and the protein matrix, the approach of two oxygen atoms to form the essential O−O bond, the movement of protons due to deprotonation reactions, and the eventual release of the product (O_2). The second type of structural effect, i.e., static effects, arises from structural modifications of the protein matrix and its encapsulated prosthetic groups by phenomena such as hydrogen bond formation/breakage, changes in the electrostatic interactions due to pH effects, and the opening/closing of essential structural elements, such as S−S bridges, etc.

There is now growing information on structural events taking place within the WOC and its regulatory subunits. Recently, FTIR experiments revealed that structural changes (probably in the ligand sphere) are coupled with the redox transition $S_1 \rightarrow S_2$ (Noguchi et al., 1992). A significant change in the nuclear geometry was inferred to take place

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[1] Abbreviations: α and β, probabilities of misses and double hits, respectively, of the redox transitions in the water-oxidizing complex; Hepes, *N*-(2-hydroxymethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-morpholinoethanesulfonic acid; NH_2R, redox active amine (R = OH (hydroxylamine), NH_2, (hydrazine)); PS II, photosystem II; S_i state, redox state of the water-oxdizing complex, with i = number of stored oxidizing redox equivalents; S_1, dark relaxed redox state in normal thylakoids; t_{inc}, dark incubation time; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; Y_D, redox active tyrosine of polypeptide D2; WOC, water-oxidizing complex.

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during the $S_2 \rightarrow S_3$ transition, on the basis of an analysis of the temperature dependence of the redox transitions $S_i \rightarrow S_{i+1}$ within the framework of classical Marcus theory [for reviews, see Marcus and Sutin (1985) and de Vault (1984)] for thermally activated, nonadiabatic electron transfer (Renger & Hanssum, 1992). This conclusion is supported by the finding of a marked difference between redox states S_2 and S_3 of the WOC with respect to their reaction with hydrophilic amine type reductants like NH_2NH_2 and NH_2OH (Messinger & Renger, 1990; Messinger et al., 1991).

In addition to these dynamic structural changes, coupled with particular redox transitions within the WOC, there is now growing evidence for "static" structural changes that are relevant for the functional reaction pattern of the WOC. On the basis of the effect of D_2O on the thermal stability of O_2 evolution in thylakoids and PSII membrane fragments, 6−7 hydrogen bonds were inferred to be essential for the functional integrity of the WOC (Renger et al., 1989, 1994). In terms of pH-induced effects, a thorough analysis of the interaction between the WOC in different S_i states and other endogenous redox groups (Y_D/Y_D^{ox}, Q_B/Q_B^-) led to the conclusion that marked structural changes take place near pH 5.0 and between pH 6.5 and 7.0 (Messinger & Renger, 1994). Furthermore, there are independent lines of evidence for another marked conformational change at around pH 7.4 (Völker et al., 1985; Packham et al., 1982; Lindberg et al., 1993).

The present study is an attempt to unravel pH-induced structural changes in the WOC by an independent assay. The reactivity of the WOC in its dark relaxed state S_1 toward the exogenous hydrophilic reductants NH_2NH_2 and NH_2OH was used as an indicator to reflect structural determinants of functional relevance. Both substances are protonizable, and therefore the reactivity also depends on their states of protonation. However, as both compounds differ by nearly 2 pH units in their pK values, it is possible to distinguish between effects arising from direct protonation of the species [see Beck and Brudvig (1988)] and phenomena due to pH-induced structural changes in the WOC (Messinger & Renger, 1994). The results presented here provide further evidence for the "static" type of pH effects. On the basis of these findings and data reported in the literature, three structural transitions were inferred to take place in the WOC at pH values of around 5.3−5.6, 6.2−6.5, and above 7.4.

MATERIALS AND METHODS

Thylakoids were prepared from market spinach according to a procedure similar to that described by Winget et al. (1965). These samples were enriched with Y_D^{ox} according to Messinger and Renger (1990).

The flash-induced O_2 oscillation patterns were measured with a modified Joliot type electrode (Joliot, 1972) that kept the temperature of the buffer reservoir and the electrode constant to within ±0.3 °C. The measurements were performed at an electrode temperature of 7 °C. As the electrode system does not allow rapid injection of the reactants, the following procedure was used: 80 μL of the sample containing thylakoids in the redox state $Y_D^{ox}S_1$ suspended in a buffer of 0.3 M mannitol, 20 mM $CaCl_2$, 10 mM $MgCl_2$, and 50 mM Mes/NaOH, Hepes/NaOH, or Tricine/NaOH for pH intervals of 5.0−6.8, 6.8−7.8, or 8.0−8.6, respectively, was mixed with 20 μL of a buffer solution with different concentrations of NH_2OH or NH_2NH_2. All solutions were kept on ice until use. It must be emphasized that the NH_2R solutions are not stable, especially at extreme pH values, and that changes in the actual NH_2R concentration are the most important source of experimental errors in the determination of the rate constants of NH_2R-induced S_i state reduction. Therefore, only freshly prepared NH_2R solutions are used in order to minimize this type of error. Before mixing, the thylakoid suspension and the amine-containing solution both were independently adjusted to the pH values given in the figure legends. After this treatment, 10 μL of the suspension was rapidly transferred in the dark to the bare platinum cathode of the Joliot type electrode, and the polarization (−0.75 V) was switched on about 20 s before the measurement. This procedure required about 1 min, so that the total dark incubation of the state $Y_D^{ox}S_1$ was given by the storage time on ice plus the time gap of 1 min between sample transfer and measurement.

The deconvolution of the measured oxygen yield data into S_i state populations was performed with a fit program based on the formulas

$$\vec{S}_n = \mathbf{K}\vec{S}_{n-1}\delta \tag{1}$$

and

$$Y_n = (1 - \alpha)[S_3]_{n-1} + \beta[S_2]_{n-1} \tag{2}$$

where $\vec{S}_{n-1}$ and $\vec{S}_n$ are vectors of the S_i state populations before and after the nth flash of the train, $\mathbf{K}$ is the extended Kok matrix [for details, see Messinger et al. (1991)], Y_n is the oxygen yield due to the nth flash; and δ is an activity parameter that compensates for an increase or decrease in the number of oxygen-evolving centers during the experiment. This activity parameter is relevant only under special conditions (see Results). The S_i state vectors used here also comprise formal redox states below S_0, i.e., S_{-1}, S_{-2}, and S_{-3}. The assumption of an S_{-3} state often leads to a marked improvement of the fit that cannot be achieved by other means, e.g., the variation of the miss or double-hit parameters [see also Messinger et al. (1991)]. However, it must be emphasized that, in contrast to S_{-2}, convincing evidence for the existence of a stable S_{-3} is still lacking because sufficiently high populations of this state have not yet been achieved (Messinger & Renger, 1993). As the existence of S_{-3} is irrelevant for the conclusions of this study, it will be used here only as a fit parameter. In agreement with our previous studies, the parameters of misses (α) and double hits (β) are assumed to be the same for all S_i state transitions and independent of varying NH_2R concentrations or incubation times. These parameters were determined in control samples for each set of measurements at the corresponding pH. The S_i state populations after NH_2R incubation were then determined by minimization of the square mean deviation by systematic manual variation of the initial S_i state population. The rate constants for the S_1 state reduction by NH_2R, $k_{S_1}(NH_2R)$, were obtained from semilogarithmic plots of the fitted S_1 state populations, $[S_1]$, as a function of the product $t_{inc}[NH_2R]$, where $[NH_2R]$ is the total concentration of NH_2R and t_{inc} is the total incubation time.

A last point must be addressed briefly. An extensive treatment of thylakoids with either NH_2NH_2 (at rather long incubation times) or NH_2OH (at high concentrations) gives rise to anomalous amperometric signals overlapping with

those that are due to flash-induced oxygen evolution via the conventional Kok cycle (see Results). This artifact was eliminated in the following way. The signals caused by the 2nd and 3rd flashes entirely reflect the artifact because no oxygen evolution can occur due to a virtually complete dark population of redox states below S_0 in NH_2OH-(NH_2NH_2-) treated samples (this is confirmed by the absence of these signals under milder incubation conditions). Therefore, the amplitudes of these two signals, which are very similar in size (in contrast, the signal due to the first flash is larger), were averaged and subtracted from the amplitudes of all signals following in the sequence. The amplitudes corrected in this way were then fitted within the framework of eqs 1 and 2.

RESULTS

As the mechanisms of S_i state reduction by NH_2R at neutral pH markedly differ for NH_2OH (1-electron reaction sequence) and NH_2NH_2 (2-electron reaction sequence) [see Messinger et al. (1991)], it first remains to be shown whether this characteristic feature is widely independent of pH. Two parameters are suitable indicators for 2-electron type mechanism: (i) the occurrence of a binary oscillation at intermediate incubation times, where about 50% of the centers have been reduced to S_{-1} and the nonreacting fraction still remains in S_1, and (ii) the O_2 yield due to the 5th flash exceeds that of the 6th flash after prolonged incubation (Messinger et al., 1991). In marked contrast to the features of a 2-electron reduction, the 1-electron mechanism induced by NH_2OH leads (i) to a transient rise in the O_2 yield of the 4th flash (i.e., the S_0 population) and (ii) to a lower ratio of Y_5/Y_6 at long incubation times or high NH_2OH concentrations.

In order to check whether these features of the NH_2NH_2- and NH_2OH-mediated effects are dependent on pH, the oscillation patterns of the oxygen yield were measured at pH 5.0 and 8.0 in dark-adapted spinach thylakoids incubated on ice at these pH values either with NH_2NH_2 or NH_2OH. In the case of NH_2NH_2, the concentration was kept constant and various incubation times were tested to monitor the S_i state kinetics, whereas when using NH_2OH the concentration was varied at a constant incubation time of 90 s because this procedure permits better resolution of the reactivity [for details, see Messinger et al. (1991)]. The data obtained are presented in Figures 1 and 2. In control samples, slight pH-dependent differences are observed between pH 5.6 and 8.0, corresponding with measurements reported recently (Messinger & Renger, 1994). In the case of NH_2NH_2, binary oscillations at intermediate incubation times and high O_2 yields induced by the 5th flash after sufficiently long incubation times are observed at both pH 5.6 and 8.0 (see traces c−f in Figure 1). Although these effects are (mainly due to the larger miss parameters at these pH values) not as pronounced as those at neutral pH, these findings suggest that the 2-electron mechanism of NH_2NH_2 is not significantly influenced by pH in the range $5.6 < pH < 8.0$. The data from experiments performed with NH_2OH are compiled in Figure 2. In agreement with previous results obtained at pH 7.2 (Messinger et al., 1991), the 1-electron reduction mechanism appears to be dominant at both pH values (5.6 and 8.0). This is clearly illustrated by the significant rise in the O_2 yield of the 4th flash and the lack of binary oscillations at intermediate NH_2OH concentrations, together with the relatively lower ratio of the O_2 yields due to the 5th and 6th

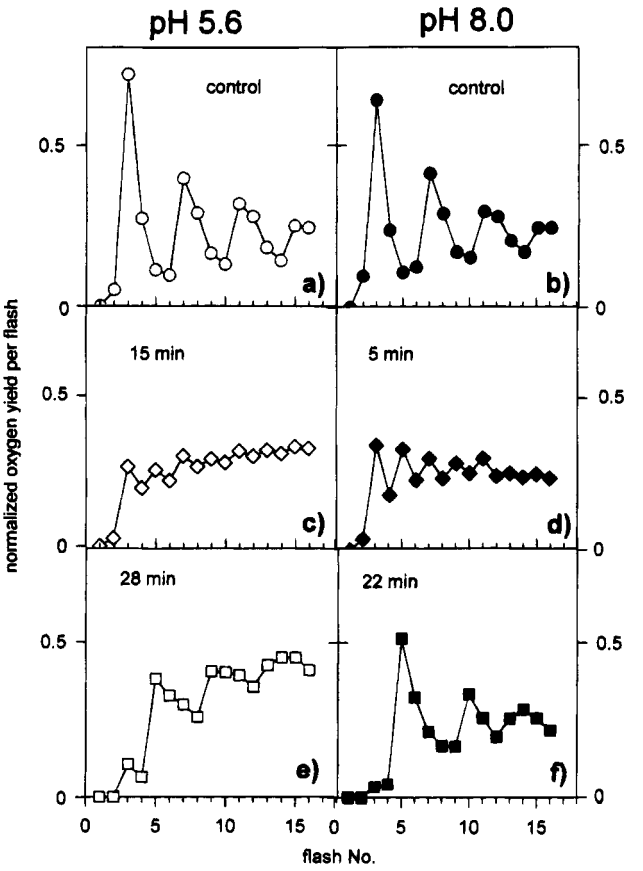

FIGURE 1: Normalized oxygen yield per flash as a function of the number of saturating single-turnover flashes in spinach thylakoids after different times of dark incubation with 6 and 0.5 mM NH_2-NH_2 at pH 5.6 and 8.0, respectively. The incubation times are given in the figure. For other experimental details, see the Materials and Methods.

flashes (Y_5/Y_6) at higher concentrations (compare Figures 1d and 2d and 1f and 2f, respectively).

In order to obtain titration curves of the pH effect, analogous experiments were performed at steps of 0.2 pH unit throughout the range $5.2 \leq pH \leq 8.6$. From these measurements, the rate constants for the reactions $S_1 \rightarrow S_{-1}$ in the case of NH_2NH_2 and $S_1 \rightarrow S_0 \rightarrow S_{-1}$ for NH_2OH have been extracted as a function of pH by semilogarithmic plots of the normalized populations of redox state S_1, [S_1], as a function of the product $[NH_2R]t_{inc}$. Figure 3 shows the data for three characteristic pH values. The rate constant $k_{S_0}(NH_2OH)$ was determined by a mathematical fit procedure, as described in Messinger et al. (1991). From the scatter of the data points in Figure 3, the relative error of each rate constant is shown to be within the range ±15%, independent of the absolute value of $k_{S_1}(NH_2R)$ [see also Messinger et al. (1991)]. The rate constants $k_{S_1}(NH_2NH_2)$, $k_{S_1}(NH_2OH)$, and $k_{S_0}(NH_2OH)$ determined at different pH values are compiled in Table 1.

Before we deal further with these data in terms of a pH-dependent reactivity of S_1 with NH_2R, three other phenomena arising from these measurements are worth mentioning: (1) At low pH values (around 5.6) (see Figures 1 and 2), the average oxygen yield per flash increases slightly during the flash train in the NH_2R-incubated samples, but not in the corresponding control samples without NH_2R. This effect has been considered by introducing a special activity parameter, δ, into the fitting procedure of the data (for details,

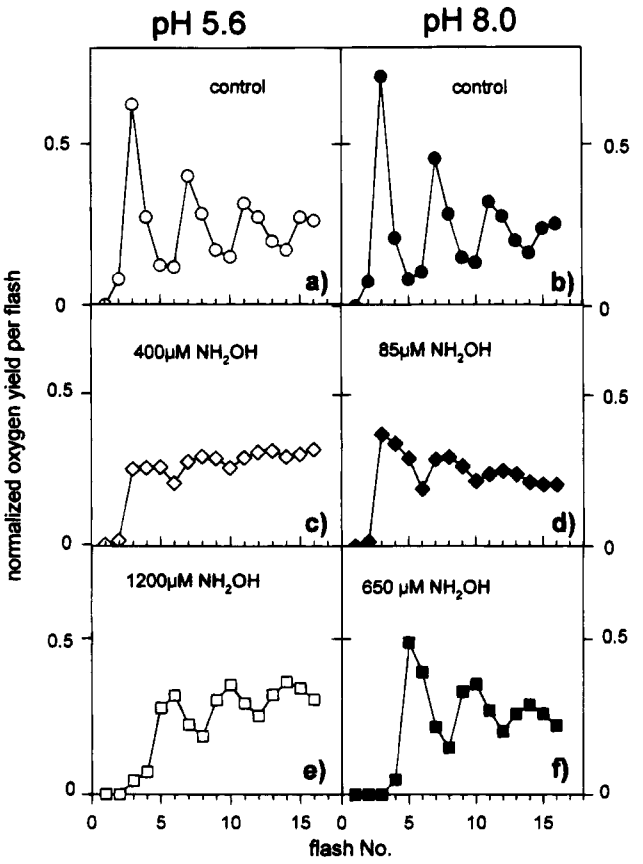

FIGURE 2: Normalized oxygen yield per flash as a function of the number of saturating single-turnover flashes in spinach thylakoids after 90 s of dark incubation with different NH_2OH concentrations at pH 5.6 and 8.0. The NH_2OH concentrations used are given in the figure. For other experimental details, see the Materials and Methods.

see Material and Methods). It remains to be clarified whether this phenomenon is due to photoactivation of the fraction of water-oxidizing complexes that become damaged by incubation with NH_2R [for a discussion, see Tamura and Cheniae (1987)]. (2) At very long incubation times with NH_2NH_2, or at high NH_2OH concentrations, amperometric signals due to the first flashes have been observed, especially at pH values near the acidic and alkaline edges of the range analyzed in this study. It is very likely that these signals arise from modified PSII centers, which do not contribute to the "normal" pattern. The amplitudes of these signals are almost constant for all flashes except for the first one (Taoka et al., 1993; Messinger et al., in preparation). These signals are not ascribed to oxygen formation via the Kok cycle and therefore have been excluded from the fitting procedure. (3) At long incubation times in the presence of NH_2NH_2, or at constant times at high NH_2OH concentrations, "superreduced" S_i states in addition to S_{-1} are formed due to subsequent reactions of S_{-1} with NH_2OH and NH_2NH_2. It was shown that, apart from the well-characterized state S_{-2} (Renger et al., 1990; Messinger et al., 1991; Messinger & Renger, 1993), the assumption of a formal redox state S_{-3} rather than an increase in the miss and double-hit parameters led to the best fits of the whole oxygen yield oscillation patterns (16 flashes) (Messinger et al., 1991). The data of this study show that for both NH_2R species the values

Table 1: Rate Constants of NH_2R-induced reduction of redox states S_1 (NH_2NH_2, NH_2OH) and S_0 (NH_2OH) as a function of pH

	NH_2OH		NH_2NH_2
pH	$k_{S_1}(NH_2OH)$ ($M^{-1}\,s^{-1}$)	$k_{S_0}(NH_2OH)$ ($M^{-1}\,s^{-1}$)	$k_{S_1}(NH_2NH_2)$ ($M^{-1}\,s^{-1}$)
5.0	15	25	
5.2	17	32	0.07
5.4	22	40	0.09
5.6	26	49	0.15
5.8	26	37	0.21
6.0	36	57	0.24
6.2	47	63	0.36
6.4	58	87	0.69
6.6	64	87	1.1
6.8	67	97	0.9
7.0	82	147	1.4
7.2	58	90	1.5
7.4	67	117	1.7
7.6	66	100	2.4
7.8	102	167	3.4
8.0	114	163	4.3
8.2	(71)	(117)	8.6
8.4	(94)	(183)	9.0
8.6			25

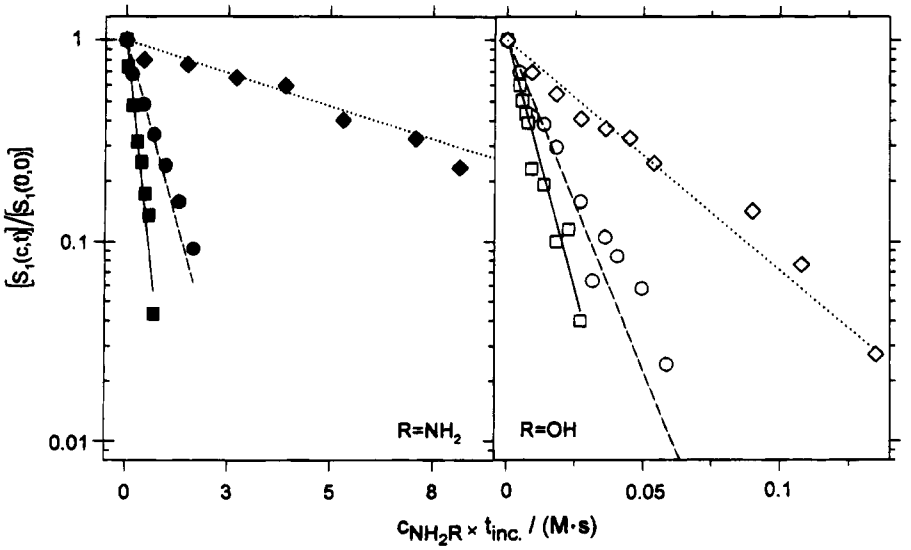

FIGURE 3: Normalized S_1 state population as a function of the product of dark incubation time, t_{inc}, and NH_2R concentration in the suspension at pH 5.6 (◆, ◇), pH 7.4 (●, ○) and pH 8.0 (■, □): left traces, R = NH_2; right traces, R = OH. For further details, see the Materials and Methods and text.

of the S_{-2} populations achieved and of the fit parameter S_{-3} are—in a complex way—dependent on pH. As this phenomenon does not influence the values of $k_{S_1}(NH_2R)$, it will not be discussed further in this study.

The data summarized in Table 1 reveal that the ratio of the corresponding rate constants of S_1 reduction, $k_{S_1}(NH_2OH)/k_{S_1}(NH_2NH_2)$, is strongly dependent on pH, although the different types of reaction mechanisms induced by NH_2OH and NH_2NH_2, respectively, are mainly conserved over the pH range analyzed in this study (vide supra). Values of 243 and 27 were found at pH 5.2 and 8.0, respectively, i.e., the ratio decreases by a factor of about 9 in this pH range. At first glance, one might assume that this difference is simply due to the different pK_a values of NH_2OH and NH_2NH_2, provided that only the unprotonated species are reactive as proposed previously (Beck & Brudvig, 1988). To check whether this idea provides a satisfying explanation of the experimentally observed values of $k_{S_1}(NH_2OH)/k_{S_1}(NH_2NH_2)$, the concentration ratio of the unprotonated forms of both species, i.e., NH_2OH and NH_2NH_2, was calculated at pH 5.2 and 8.0. The fraction of the unprotonated form NH_2R, i.e., of the conjugate of base NH_3^+R, X_{ba}, normalized to the total concentration of the amines is obtained according to the formula

$$X_{ba} = \frac{1}{1 + 10^{pK_a - pH}} \quad (3)$$

where pK_a is the corresponding pK value of NH_2NH_2 or NH_2OH, and pH refers to the aqueous bulk phase of the thylakoid suspension. Extrapolation of literature data (Bissot et al., 1957; Condon et al., 1974; Lumme et al., 1965; Ware et al., 1936) to our experimental conditions (temperature θ = 2−4 °C, ionic strength $I \approx 115$ mM) leads to actual pK_a values of about 8.6 (± 0.10) and 6.6 (± 0.10) for NH_2NH_2 and NH_2OH, respectively. Insertion of these data into eq 3 leads to the result that the ratio of the unprotonated forms, $X_{ba}(NH_2OH)/X_{ba}(NH_2NH_2)$, increases by a factor of about 20 in the pH range from 5.2 to 8.0. Accordingly, the ratio $k_{S_1}(NH_2OH)/k_{S_1}(NH_2NH_2)$ is expected to increase by the same factor of about 20 if only the unprotonated forms of both amines act as the active species. A comparison with the experimentally determined value of <10 reveals that the simple model does not permit a satisfying explanation, and therefore it appears to be worth analyzing the data in more detail.

Figure 4 depicts the rate constants $k_{S_1}(NH_2NH_2)$ and $k_{S_1}(NH_2OH)$ as a function of pH. A comparison of the general shapes of the pH dependencies of $k_{S_1}(NH_2OH)$ and $k_{S_1}(NH_2NH_2)$ reveals a very interesting phenomenon: both traces exhibit strikingly common features, despite the quantitative differences in the rate constants and the reaction mechanisms discussed earlier. Three transitions are discernible as is shown by the full line that simply connects the data points: (i) a small, but reproducible step around pH 5.2−5.8, (ii) a more pronounced increase between 6.0 and 7.0, and (iii) a steep increase in the rate constants above a critical value of pH 7.4 (in the case of NH_2OH, no reliable measurements could be obtained above pH 8.0 because of the instability of both the NH_2OH solution and the sample in presence of NH_2OH). A closer inspection of the data shows that, in the case of NH_2OH, the former two transitions (i and ii) occur at slightly lower pH values compared to

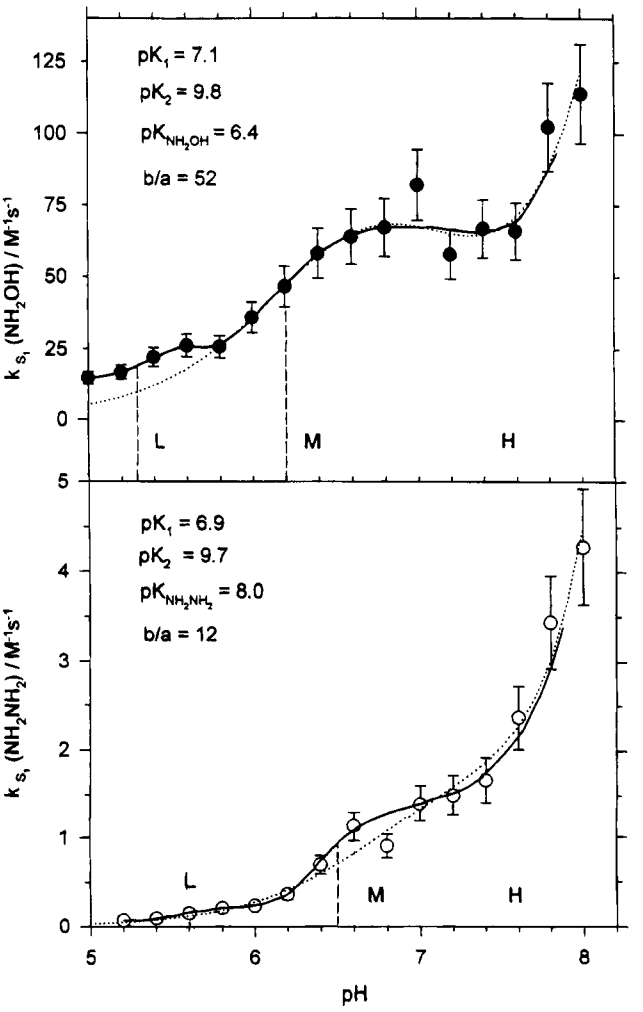

FIGURE 4: Rate constant of the NH_2R-induced dark decay of S_1, $k_{S_1}(NH_2R)$, as a function of pH: top trace, R = OH; bottom trace, R = NH_2. For calculations of $k_{S_1}(NH_2R)$, see Figure 3 and text. Numerical fits with eq 4 of the rate constants $k_{S_1}(NH_2NH_2)$ and $k_{S_1}(NH_2OH)$ as a function of pH are represented by dotted curves. The parameters used for the calculation of these curves are given in the figure.

NH_2NH_2. The apparent pK_a values are 5.3 and 6.2 for NH_2OH and 5.6 and 6.5 for NH_2NH_2. Interestingly, a very similar pH dependence is found for the rate constant of the reaction $S_0 \rightarrow S_{-1}$ induced by NH_2OH. The absolute rates of these transitions are somewhat higher than those of the $S_1 \rightarrow S_0$ reduction. The latter finding is in agreement with previous results obtained at pH 7.2 (Messinger et al., 1991). The data presented here show that this difference prevails over the entire pH range from 5.0 to 8.0 (see data in Table 1).

DISCUSSION

Qualitative Analysis of the pH Dependence of the NH_2OH and NH_2NH_2 Induced Kinetics of S_1 Reduction in Spinach Thylakoids. Before we discuss the implications of the results reported in this study, possible effects must be considered that might arise for the actual pH and NH_2R concentration near the WOC due to its separation from the outer aqueous phase by the thylakoid membrane. The experiments were performed with thylakoids that were frozen for storage and rethawed. This procedure leads to a rather leaky membrane, so that the excitation with a train of a few flashes does not

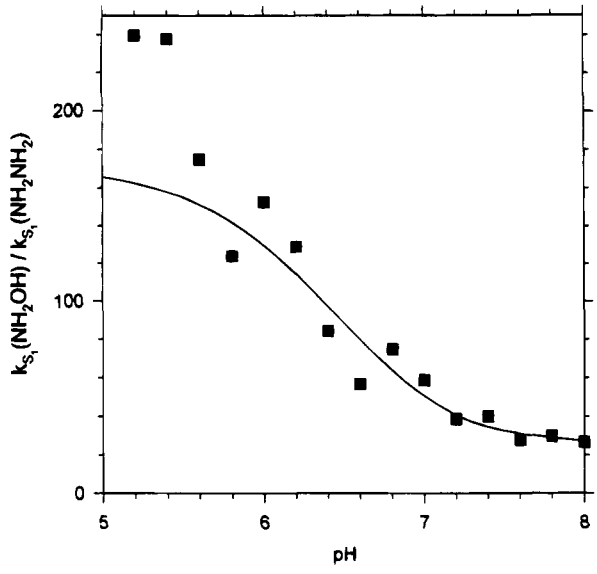

FIGURE 5: Calculated ratio $k_{S_1}(NH_2OH)/k_{S_1}(NH_2NH_2)$ as function of pH (solid line) and experimental data taken from Table 1. The values of $pK(NH_2R)$, pK_1, pK_2, and *b/a* are the same as in Figure 4.

lead to a significant ΔpH even at alkaline pH, as was shown recently by experiments with the uncoupler gramicidin (Messinger & Renger, 1994). The distribution of amines between the lumen and the outer aqueous phase depends on the transmembrane ΔpH (Schuldiner et al., 1972). In the absence of a ΔpH, the amine concentration should be virtually the same on both sides. This idea is supported by the similarity of the rate constants measured for the NH_2OH-induced S_1 reduction in isolated thylakoids (Messinger et al., 1991) and PS II membrane fragments (Beck & Brudvig, 1988; J. Messinger and G. Renger, unpublished results). On the basis of the above-mentioned consideration, it is inferred that the separation by the thylakoid membrane does not lead to significant H^+ and NH_2R concentration differences between the outer aqueous phase and the lumenal environment of the WOC, especially under the dark incubation conditions used for the NH_2R treatment (see Materials and Methods).

With respect to structure−function relations in the WOC (and associated regulatory subunits), the most interesting results of this study emerge from the pH dependencies of the rate constants of NH_2NH_2- and NH_2OH-induced S_1 decay. If one takes into account the data uncertainties (see error bars in Figure 4), two striking features are observed: (a) the rate constants $k_{S_1}(NH_2NH_2)$ and $k_{S_1}(NH_2OH)$ exhibit surprisingly similar dependencies on pH, and (b) the pH titration curves of $k_{S_1}(NH_2NH_2)$ and $k_{S_1}(NH_2OH)$ are characterized by three similar transitions at pH 5.6(5.3), 6.5(6.2), and above a critical value of 7.4. These findings readily show that they cannot be explained solely by the assumption that the unprotonated forms NH_2NH_2 and NH_2OH are the reactive species whereas the protonated forms $[N_2H_5]^+$ and $[NH_3OH]^+$ are inactive, as suggested previously (Beck & Brudvig, 1988). If this were the case, simple titration curves around the pK_a values of 8.6 (NH_2NH_2) and 6.6 (NH_2OH) would have been anticipated. This expectation is in marked contrast to the experimental findings (see Figure 4). For a more detailed interpretation of the data, two additional results reported recently must be taken into account: (i) in untreated control samples the water oxidase activity of isolated spinach thylakoids illuminated by a short flash train is almost constant within the range pH 5.0−8.0, as reflected by measurements of the average oxygen yield per flash [see Messinger and Renger (1994) and references therein], and (ii) the effects of NH_2R on the oscillation patterns of the oxygen yield per flash in dark-adapted samples are most likely caused by direct interactions between these exogenous electron donors and the manganese-containing hole storage unit [see Messinger and Renger (1993) and references therein].

On the basis of these findings, the overall shape of the pH titration curves in Figure 4 can be interpreted to reflect pH-induced structural changes in the fully competent water oxidase, which affect the reactivity of the manganese-containing hole storage unit to NH_2R. On the other hand, the ratio of the rate constants as a function of pH, $k_{S_1}(NH_2OH)/k_{S_1}(NH_2NH_2)$, is very likely related to the differences between NH_2OH and NH_2NH_2 in terms of structure, pK_a values, and reaction mechanism (1-electron transfer vs 2-electron transfer). These species-specific effects will not be discussed here.

Different lines of experimental evidence support the idea of pH-induced structural changes that modulate the properties of the water oxidase. Recently, the kinetics of S_2 and S_3 reduction by the endogenous electron donors Y_D and Q_B was analyzed as a function of pH (Renger et al., 1992; Messinger & Renger, 1994). On the basis of these data, two conformational changes of functional significance were inferred to take place: one around pH 5.0−5.5 and another with a characteristic pH_c of 6.5−7.0. Taking into account these findings and the data presented in this study, it appears reasonable to conclude that the reactivity of the water oxidase in redox state S_1 with exogenous hydrophilic electron donors like NH_2R are modulated markedly by three structural changes induced by pH. They will be referred to as L, M, and H (low, middle, and high pH, respectively). These structural changes occur at the following characteristic pH values: L between 5.3 and 5.6, M between 6.3 and 6.5, and H above a critical pH of about 7.4.

A comparison of the data gathered from two entirely different types of approaches [S_2/S_3 reduction by endogenous donors Y_D and Q_B [see Renger et al. (1992) and Messinger and Renger (1994)] vs S_1 reduction by NH_2R (this study)] reveals a striking similarity in the proposed structural changes L and M. Therefore, it can be concluded that the same changes not only influence the reactivity of the water oxidase in redox states S_2 and S_3 with the endogenous electron donors Y_D and Q_B, but also affect in a similar manner the interaction of the WOC in redox state S_1 with exogenous reductants. The proposed L change occurring between pH 5.0 and 5.5 correlates (i) with a transition from high-potential cytochrome *b*559 to its low-potential form as reported recently (Crofts & Horton, 1990) and (ii) with a drastic impairment of electronic coupling between Y_D and the water oxidase in redox states S_2 and S_3 (Renger et al., 1992; Messinger & Renger, 1994). Likewise, the proposed M transition at pH 6.5 corresponds with a marked effect on the pH dependence on the lifetimes of S_2 and S_3 (Renger et al., 1992; Messinger & Renger, 1994). Furthermore, the present data permit a clear distinction between the effect occurring around pH 6.5 (M transition) and that with a characteristic pH_c of about 7.4 (H transition). Above this pH is observed not only a drastic increase in the rate constants for S_1 reduction by NH_2R (this study) but also a loss of Cl^- (Lindberg et al.,

1993) and an increase in CN^- inhibition in Cl^--deficient medium (Packham et al., 1982). In addition, the sensitivity of the water oxidase toward an attack by trypsin drastically increases above pH 7.3−7.5 (Völker et al., 1985) and the inhibition by SO_4^{2-} becomes enhanced. This effect is also assumed to be responsible for an easier displacement of Cl^- at pH 7.5 than at pH 6.0 (Sandusky et al., 1983). At first glance, the latter finding might suggest that the increase in $k_{S_1}(NH_2R)$ is primarily caused by the loss of Cl^- above pH 7.4. This could support the reaction of NH_2R with the WOC at a Cl^- binding site, as proposed previously for NH_3 and NH_2OH (Beck & Brudvig, 1988). However, it must be emphasized that the experiments in this study were performed in the presence of 20 mM $CaCl_2$ and 10 mM $MgCl_2$. Therefore, it appears reasonable to assume that the release of Cl^- and its possible replacement by OH^- (CN^-, SO_4^{2-}) is not the trigger for the conformational change above pH 7.4 but reflects one of its consequences occurring at low Cl^- concentrations.

Possible Mechanisms for the Changes in the Rate Constants of NH_2R-Induced S_1 Reduction. In principle, two possible mechanisms have to be considered in order to explain the data reported in this study: (a) protonation of amino acids that directly (by Coulombic and/or steric effects) or via allosteric type effects impair the accessibility of the proposed binding site X in the WOC [see scheme I in Messinger et al. (1991)] and (b) a direct protonation effect on site X that diminishes either the binding affinity of NH_2R or the rate of electron transfer from NH_2R to the Mn cluster (component C in the above-mentioned scheme I) by introducing charges or by changing distances and/or mutual orientations of the reactants. For a quantitative analysis of the data, the protonation of NH_2R also has to be taken into account, because this gives rise to a shift in redox potential, the formation of a positively charged species, and an increase of the size of NH_2R. Each of these effects can result in a decrease in the rate constants via the above-mentioned mechanisms.

On the basis of these considerations, a rather simple model is used in an attempt to describe the experimental data quantitatively. It comprises three assumptions: (i) the unprotonated form of NH_2R is the reactive species, (ii) the reactivity toward NH_2R of the WOC in redox state S_1 depends on protonizable groups that are characterized by two pK_a values symbolized by pK_1 and pK_2 (a third group probably responsible for the proposed L change in the range pH 5.3−5.6 is not explicitly taken into account because this phenomenon is less pronounced in the pH titration curve than the other two transitions); and (iii) in terms of interaction with NH_2R, the most reactive state of the WOC is attained if the group(s) with pK_1 is (are) protonated and those with pK_2 are deprotonated. Accordingly, one obtains, for the rate constant $k_{S_1}(NH_2R)$,

$$k_{S_1}(NH_2R) = \frac{1}{1 + 10^{[pK_a(NH_2R) - pH]}}\left[\frac{a}{1 + 10^{(pH - pK_1)}} + \frac{b}{1 + 10^{(pK_2 - pH)}}\right] \quad (4)$$

The first factor describes the normalized concentration of the unprotonated NH_2R form (see eq 3), and the second factor (in brackets) describes the fractions of the highly reactive and less reactive states of the WOC in terms of S_1 reduction by NH_2R, where a and b are rate constants reflecting the relative reactivities of the WOC in the different protonation states.

The numerical fits of the experimental data with eq 4 are shown in Figure 4 as dashed curves. Several procedures have been used (nonlinear regression according to Levenberg−Marquart, multidimensional trisection, Monte Carlo method). All programs lead to very similar values for the fit parameters. These analyses clearly show that a satisfactory description can be achieved within the errors of the fits ($\Delta pK = \pm 0.2$). The same values of pK_1 and pK_2 are obtained for both reductants, i.e., $pK_1 = 6.9$ (7.1) and $pK_2 = 9.7$ (9.8) for NH_2NH_2 (NH_2OH). Likewise, a satisfactory fit of $k_{S_0}(NH_2OH)$ as a function of pH is achieved with virtually the same values of pK_1 (7.1) and pK_2 (9.8) that was obtained for $k_{S_1}(NH_2OH)$ (data not shown). The findings strongly support the idea that the same structural changes at and/or near the WOC induced by different protonation states modulate the accessibility toward reduction by NH_2R.

The $pK_a(NH_2R)$ was also used as a free running parameter in the above-mentioned numerical fit programs applied in this study. Values of $pK_a(NH_2NH_2) = 8.0 \pm 0.2$ and $pK_a(NH_2OH) = 6.3 \pm 0.2$ were obtained with different procedures. These data agree reasonably well with the corresponding values calculated for solutions, i.e., $pK_a(NH_2NH_2) = 8.6 \pm 0.1$ and $pK_a(NH_2OH) = 6.6 \pm 0.1$ (see Results). This finding provides additional evidence for the reliability of the fitting procedures and simultaneously shows that the protolytic equilibria of NH_2R are not markedly affected by the protein matrix. Equation 4 also permits a satisfactory description of the pH dependence of the ratio of the rate constants $k_{S_1}(NH_2OH)/k_{S_1}(NH_2NH_2)$, as is shown in Figure 5. Of course, deviations arise below pH 5.5, which are ascribed to the effect of the L transition that was not considered in eq 4.

CONCLUSIONS

The data presented in this study provide strong evidence for pH-induced structural changes at and/or near the water-oxidizing complex (WOC) that modulate its reactivity with NH_2NH_2 and NH_2OH. Three different transitions were discernible in the titration curves of the rate constant $k_{S_1}(NH_2R)$ for the NH_2R (R = OH, NH_2) induced S_1 reduction at pH 5.3−5.6, 6.2−6.5, and above 7.4. Similar structural changes were recently inferred to modulate other activities of the WOC, such as the interaction of S_2 and S_3 with Y_D, the susceptibility to proteolytic attack by trypsin, or the binding and exchange of Cl^-. Therefore, it appears reasonable to assume that these pH-dependent transitions are a general feature of the WOC and/or its regulatory subunits. This phenomenon is basically independent of the redox state S_i of the WOC. Therefore, the protonizable groups are likely to be somewhat distant from the hole storage unit. It appears tempting to correlate the apparent pK-values derived from the curve-fitting procedure using eq 4, i.e., pK_1 and pK_2, with protonations of particular amino acid residues of the protein matrix. Histidines and tyrosines seem to be the most likely candidates. At the current stage of knowledge a more specific assignment cannot be achieved. Further experiments are required to identify the presumed amino acid residues.

REFERENCES

Beck, W. F., & Brudvig, G. W. (1988) *J. Am. Chem. Soc. 110,* 1517−1523.

Bissot, T. C., Parry, R. W., & Campell, D. H. (1957) *J. Am. Chem. Soc. 79,* 796−800.

Bouges, B. (1971) *Biochim. Biophys. Acta 234,* 103−112.

Condon, F. E., Reece, R. T., Shapiro, D. G., Thakkar, D. C., & Goldstein, T. B. (1974) *J. Chem. Soc., Perkin Trans. 2,* 1112−1121.

Crofts, J., & Horton, P. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, Ed.) Vol. 1, pp 391−394, Kluwer, Dordrecht, The Netherlands.

Debus, R. J. (1992) *Biochim. Biophys. Acta 1102,* 269−352.

De Vault, D. (1984) *Quantum Mechanical Tunneling in Biological Systems,* 2nd ed., Cambridge University Press, Cambridge.

Joliot, P. (1972) *Methods Enzymol. 24,* 123−134.

Kok, B., Forbush, B., & McGloin, M. (1970) *Photochem. Photobiol. 11,* 457−475.

Lindberg, K., Vänngard, T., & Andréasson, L.-E. (1993) *Photosynth. Res. 38,* 401−408.

Lumme, P., Lahermo, P., & Tummavuori, J. (1965) *Acta Chem. Scand. 19,* 2175−2188.

Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 84,* 265−322.

Messinger, J., & Renger, G. (1990) *FEBS Lett. 277,* 141−146.

Messinger, J., & Renger, G. (1993) *Biochemistry 32,* 9379−9386.

Messinger, J., & Renger, G. (1994) *Biochemistry 33,* 10896−10905.

Messinger, J., Wacker, U., & Renger, G. (1991) *Biochemistry 30,* 7852−7862.

Noguchi, T., Ono, T., & Inoue, Y. (1992) *Biochemistry 31,* 5953−5956.

Packham, N. K., Mansfield, R. W., & Barber, J. (1982) *Biochim. Biophys. Acta 681,* 538−541.

Renger, G. (1987) *Photosynthetica 21,* 203−224.

Renger, G. (1992) in *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., Ed.) pp 45−99, Elsevier, Amsterdam.

Renger, G. (1993) *Photosynth. Res. 38,* 229−247.

Renger, G., & Hanssum, B. (1992) *FEBS Lett. 299,* 28−32.

Renger, G., Eckert, H.-J., Hagemann, R., Hanssum, B., Koike, H., & Wacker, U. (1989) in *Photosynthesis: Molecular Biology and Bioenergetics* (Singhal, G. S., Barber, J., Dilley, R. A., Govindjee, Haselkorn, R., & Mohanty, R., Eds.) pp 357−371, Narosa, New Delhi, India.

Renger, G., Messinger, J., & Hanssum, B. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. 1, pp 845−848, Kluwer, Dordrecht, The Netherlands.

Renger, G., Messinger, J., & Wacker, U. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. II, pp 329−332, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Renger, G., Gleiter, H., Haag, E., & Reifarth, F. (1993) *Z. Naturforsch. 48c,* 392−402.

Renger, G., Bittner, T., & Messinger, J. (1994) *Biochem. Soc. Trans. 22,* 318−322.

Rutherford, A. W., Zimmermann, J.-L., & Boussac, A. (1992) in *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., Ed.) pp 179−229, Elsevier, Amsterdam.

Sandusky, P. O., Selvius DeRoo, C. L., Hicks, D. B., Yocum, C. F., Ghanotakis, D. F., & Babcock, G. T. (1983) in *The Oxygen Evolving System of Photosynthesis* (Inoue, Y., Crofts, A. R., Govindjee, Murata, N., Renger, G., & Satoh, K., Eds.) pp 189−199, Academic Press, Tokyo.

Schuldiner, S., Rottenberg, H., & Avron, M. (1972) *Eur. J. Biochem. 25,* 64−70.

Tamura, N., & Cheniae, G. M. (1987) *Biochim. Biophys. Acta 890,* 179−194.

Taoka, S., Jursinic, P. A., & Seibert, M. (1993) *Photosynth. Res. 38,* 425−431.

Völker, M., Ono, T., Inoue, Y., & Renger, G. (1985) *Biochim. Biophys. Acta 806,* 25−34.

Ware, B. C., Spulnik, J. B., & Gilbert, E. C. (1936) *J. Am. Chem. Soc. 58,* 1605−1606.

Winget, G. H., Izawa, S., & Good, N. E. (1965) *Biochem. Biophys. Res. Commun. 21,* 438−441.

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