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The reaction of H_2S with the photosynthetic water-oxidizing complex and its lack of reaction with the primary electron acceptor in spinach

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Inhibition of photosynthetic water oxidation by H_2S, a substrate analog, has been investigated using equilibrium titrations and EPR spectroscopic detection of the electron donors in spinach Photosystem II (PS II) membranes and compared to inhibition by NH_2OH. Like NH_2OH, H_2S inhibits formation of the S_2 oxidation state of the water oxidizing complex by a two-step process in the dark. Initially, reversible inhibition of S_2 occurs upon binding to a high affinity site in the dark (S_1) state at a low concentration of inhibitor (50% inhibition: 0.07 μmol H_2S/mg Chl, corresponding to about 15 H_2S/PS II). This causes no loss of steady-state O_2 evolution and can be reversed by illumination at room temperature, which causes multiple turnovers. At higher concentrations, irreversible inhibition occurs due to the cooperative release of 3 out of 4 Mn^{2+}/PS II using mild washing conditions, with parallel loss of O_2 evolution activity. This stoichiometry of Mn release is preserved throughout the entire concentration range of inhibition by both H_2S and NH_2OH, suggesting a common binding site for at least 3, and possibly all 4, of the Mn ions which are present in PS II. This is consistent with independent earlier work showing the net release of 4 Mn/PS II using stronger washing conditions, and also with EPR spectroscopic evidence assigning the S_2 multiline signal to a cluster of 3–4 Mn ions. The concentration of H_2S which induces 50% irreversible inhibition is 17-fold greater than that required for NH_2OH. Qualitatively, the weaker inhibition by H_2S is consistent with its lower oxidation potential compared to NH_2OH. However, the quantitative agreement is poor, suggesting that other environmental factors must be involved in determining their relative inhibition strengths. Unlike NH_2OH, H_2S does not affect the structure of the primary quinone electron acceptor, Q_A^--His-Fe (structure by analogy to bacterial reaction centers), as seen by formation of the normal EPR signals at $g = 1.8$ and $g = 1.9$ for the photoreduced semiquinone, instead of the shifted resonance at $g = 2.1$ observed with NH_2OH. H_2S is therefore unable to bind to the NH_2OH site on the acceptor side of PS II.

Introduction

All the substrate analog-type inhibitors of the water oxidizing complex, such as NH_2OH, NH_2NH_2, H_2O_2, etc. [1–7], that have thus far been studied in detail contain nitrogen or oxygen. These inhibitors bind to the water-oxidizing complex and alter the redox state by either reduction or oxidation of manganese. NH_2OH is the most potent of these. Although these are believed to bind directly to manganese, the evidence for this is indirect and as yet unproven.

This report presents the first in depth studies of the reaction of the sulfur containing water analog, H_2S with the water-oxidizing complex. Our aim is to see whether new insights into the unsolved

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mechanism of water oxidation might be revealed by the use of a sulfur-containing substrate analog. Earlier studies have provided a basis for this expectation, since sulfide has already been found to act as an electron transport inhibitor at an unspecified site between water and PS II in both plant chloroplasts and cyanobacterial cells [8,9]. Since selected redox data and binding constants for sulfur vs. oxygen and nitrogen ligands to free Mn^{2+} in solution are available, it is possible to ascertain which of these factors, if any, determine the effectiveness of these analogs as inhibitors. Furthermore, if H_2S can displace bound water from manganese, then a variety of spectroscopic methods which are sensitive to ligand covalency, such as EPR, electronic absorption, EXAFS, etc., could provide new structural information on the manganese site.

Materials and Methods

The preparation of PS II membranes and the assay of O_2 rate activity are the same as described previously [7]. Concentrated PS II membranes that had been frozen in a 200 mM sucrose buffer containing 30% glycerol were thawed and washed in a medium containing 200 mM sucrose/15 mM NaCl/20 mM Mes/2 mM EDTA prior to use. These gave absolute O_2 evolution rates in the range of 350–450 μM O_2/mg Chl per h.

The H_2S stock solution was prepared by starting with a crystal of $Na_2S \cdot 9H_2O$ that was 2–3 times larger than the final amount needed to make a 100 mM stock solution. This crystal was partly dissolved in deionized water to remove oxidation products on the crystal surface. The remaining crystal of pure Na_2S was then dissolved in an appropriate volume of degased deionized water. The pH of this solution was reduced from about 12.5 to about 6.8 with HCl to give a colorless solution. Lowering the pH below this gave a yellow solution that was unsuitable. This stock was used within half an hour for treatment of the PS II membranes. If the initial crystal was not cleaned, the solution became yellow after reduction of the pH to 6.8.

Both the stock and final H_2S solutions were assayed for H_2S to determine its concentration. For the stock solution, H_2S was directly assayed by iodometric titration [10]. In the range 9–50 mM, the measured value was within 20% of the value determined based on the weight of the $Na_2S \cdot 9H_2O$.

For measuring H_2S concentrations in the range 0–1 mM a direct colorimetric titration was found to be unsuitable, due to the very low concentrations involved. For measurements in this range, a large volume (1 liter) of the dilute H_2S solution was treated with $CdCl_2$ under slightly basic conditions and the precipitated CdS was filtered and redissolved in a smaller volume (50 ml) under acidic conditions. This solution was then assayed iodometrically as above. At 0.25 mM H_2S (gravimetric), the actual concentration in solution was found to be 0.18 mM, hence the actual concentration is 1.4-times lower than the gravimetric value. When air-saturated water was used to dilute the stock solution to 0.25 mM (gravimetric), the measured concentration was found to be 0.16 mM after 15 min incubation. This indicates that, within the error of the method, there was little additional consumption of H_2S by air redissolved in the 20 min incubation time used here.

For treatment with H_2S, the PS II membranes were resuspended to a chlorophyll concentration of about 1 mg/ml and dark-adapted for at least 30 min at 273 K to favor population of the S_1 state. H_2S was then added in the dark to 1 ml of the membranes and allowed to incubate for 20 min in the dark. The suspension was then pelleted at $48\,000 \times g$ for 30 min and analyzed for manganese, O_2 activity and by EPR. For EPR measurements, an alternate method was sometimes used in which incubation with H_2S at a chlorophyll concentration of 4 mg/ml was carried out directly on the EPR samples without subsequent centrifugation. The results of the two methods were the same.

Manganese was determined by atomic absorption using a graphite furnace. EPR spectra were obtained at 9.5 GHz on a Varian E-12 spectrometer operating with 10 kHz field modulation and fitted with an Oxford Instruments ESR-900 continous flow helium cryostat. The S_2 multiline EPR signal was observed at 10 K after illumination at 200 K by a tungsten source. Cytochrome *b*-559 was monitored as the oxidized low potential form by EPR at $g = 2.95$.

Results

Binding of H_2S to the S_1 state

As was the case for our previous studies of water-oxidation inhibitors [7,11], the yield of the S_2 multiline EPR signal was used as an indicator of the extent of loss or blockage upon binding in the dark to the precursor state S_1. Curve 1 in Fig. 1 shows the course of loss of the S_2 multiline EPR signal during equilibrium titration against H_2S added in the dark at 277 K. The sample was assayed by illumination at 200 K. The amount of H_2S is normalized to the amount of chlorophyll in the sample. The yield decreases monotonically;

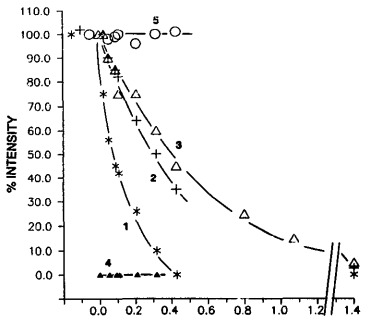

Fig. 1. The effect of H_2S on the O_2 evolution rate and the yield of EPR signals in spinach PS II membranes. Curve 1 (*): the yield of the S_2 multiline EPR signal generated by CW illumination at 200 K after incubation with H_2S in the dark for 15 min followed by centrifugation and resuspension in buffer. Curve 2 (+): yield of the S_2 multiline EPR signal produced by CW illumination at 200 K, as for curve 1, but after consuming the H_2S by photooxidation at 277 K and dark readapting. Curve 3 (△): steady-state O_2 rate after treatment as in curve 1. Curve 4 (▲): yield of the $g = 4.1$ signal for the altered S_2 state manganese center after treatment as in curve 1. Curve 5 (○): Yield of the $g = 2.95$ signal for low potential cyt *b*-559 observed in the dark. The curves are drawn to aid in distinguishing the data sets only. EPR conditions: microwave freq., 9.22 GHz; microwave power, 20 mW; modulation amplitude, 32 G; sample temperature, 10 K. Chlorophyll concentration = 3.5–4.0 mg/ml. Suspension buffer: 200 mM sucrose/20 mM Mes/15 mM NaCl (pH 6.5). Exogenous acceptor: 0.5 mM 2,5-DCBQ in 1–2% methanol.

50% loss occurs at 0.07 µmol H_2S/mg Chl and 100% loss occurs at 0.45 µmol H_2S/mg Chl (0.07 µmol H_2S/mg Chl corresponds to about 15 H_2S/PS II; one photosynthetic unit corresponds to 225 ± 25 chlorophyll) [12–14]. Evidence that some of this loss is photoreversible is given in curve 2. This shows the S_2 multiline EPR signal yield after warming the treated samples to 277 K and illuminating them with a 20 W tungsten source for 2 min, followed by a second dark adaptation for 30 min and reillumination at 200 K. This treatment consumes H_2S, apparently by photooxidation in analogy with the behavior of NH_2OH [7,11]. Irreversible loss of 50% of the signal occurs at an H_2S concentration of 0.31 µmol/mg Chl.

In untreated membranes, another EPR spectral form of the manganese center comprising the water-oxidizing complex has been reported at $g = 4.1$. It forms if the illumination is performed at 140 K instead of 200 K [15,16]. A negligible yield of this form of the S_2 state was seen in both untreated and H_2S-treated (curve 4) PS II membranes when illuminated at 200 K. Therefore, the loss of the S_2 multiline signal induced by H_2S is not replaced by the other known spectral form of the manganese center during the course of the titration. This loss of signal is therefore attributed to a loss in population of the S_2 oxidation state.

Curve 3 shows that the steady-state O_2 evolution rate closely parallels the yield of centers which retain the photoreversible multiline signal in curve 2. The difference between curve 2 and the control yield is attributed to centers which lose the S_2 multiline signal irreversibly and essentially in parallel with loss of water oxidation activity.

The reversible loss of the S_2 multiline signal seen below 0.45 µmol H_2S/mg Chl in curve 1 is not accompanied by a change in the yield of oxidized low potential cytochrome *b*-559, as observed by its resonance at $g = 2.95$ (curve 5).

The reversible loss of the multiline signal seen at low H_2S concentrations (curve 1) was sometimes not seen. The reason for this irreproducibility was not investigated in detail, but appears to have something to do with consumption of H_2S by either inadvertent illumination, residual oxidants present in the PS II membranes or dissolved O_2 in the degassed buffer. On the other hand, the titration curve for the irreversible loss of the mul-

tiline signal did not vary. Also, at pH = 6.5, where these experiments were performed, H_2S exists in equilibrium with HS^- ($pK_a = 7.0$), and so the relative concentration of these is very sensitive to the medium pH.

Release of manganese from PS II by H_2S

The irreversible losses of the multiline signal and O_2 evolution, given by curves 2 and 3 in Fig. 1, are accompanied by a parallel release of Mn from the membranes into the supernatant as monitored by the Mn retained in the pellet as shown in Fig. 2.

The total number of Mn atoms per PS II varied between 4.5 and 5.0 for different preparations. At 6.5 μmol H_2S/mg Chl O_2, the activity is completely lost and the PS II membranes still retain about 1 Mn/PS II. Thus, 3.5–4.0 Mn are susceptible to H_2S release. Comparing Fig. 2 and curve 3 of Fig. 1, we see that release of the first manganese atom at 0.03 μmol H_2S/mg Chl does not affect O_2 activity, identifying this population with extraneous Mn. This can also be removed by repeated washings with chelators [2,17,18]. Thus, of the 3.5–4 Mn which need to be present for optimal activity in these samples, a total of 2.5–3 are released by mild washing in H_2S. More Mn can be released using stronger washing conditions. Release of 3–4 Mn is also seen upon incubation with low NH_2OH concentration, the precise amount being a function of the washing conditions [7,12].

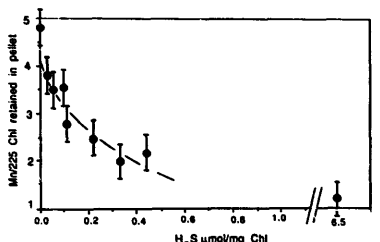

Fig. 2. Total Mn retained in the pellet after treatment with H_2S in the dark followed by centrifugation and resuspension in buffer.

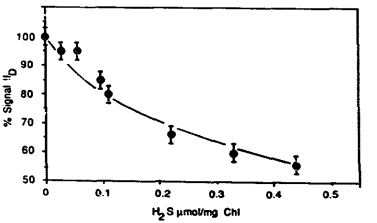

Fig. 3. The effect of H_2S concentration of the yield of EPR signal II. Chlorophyll concentration = 3.5–4.0 mg/ml, exogenous acceptor = 0.5 mM 2,5-DCBQ in dimethyl sulfoxide. EPR conditions as in Fig. 1, except microwave power = 0.1 mW and modulation amplitude = 4.0 G.

Primary electron acceptor

We have shown previously that NH_2OH reacts with PS II membranes at a second site which induces a shift of the EPR resonance of the primary quinone electron acceptor, Q_A^--His-Fe, from $g = 1.9$ to a new resonance at $g = 2.1$ [7,11]. By contrast, H_2S gave no evidence for modification of either of the acceptor signals at $g = 1.9$ or 1.82 at concentrations below 4.5 μmol H_2S/mg Chl (data not shown).

Signal II

The titration curve given in Fig. 3 shows that H_2S reacts in the dark with PS II membranes to reduce the oxidized species responsible for signal II (both dark and slow components). The signal intensity is reduced to about 55% at 0.45 μmol H_2S/mg Chl, where O_2 evolution activity is completely abolished. Comparing Figs. 2 and 3, we see that reduction of signal II by H_2S occurs in parallel with the release of manganese.

Discussion

The reversible reaction of H_2S with the water-oxidizing complex

The *X*-intercept of curve 1 in Fig. 1 indicates that a maximum of 0.45 μmol H_2S/mg Chl (86 H_2S/PS II) are required to completely eliminate formation of the S_2 multiline EPR signal. At this concentration, only 40% of the signal is recoverable: the remaining 60% is irreversibly lost (Fig. 1,

curve 2) due to the release of manganese (Fig. 2). In comparing the results from Fig. 1 with the reversible loss of the S_2 multiline signal by NH_2OH [7,11], we see that the inhibition constant for H_2S is about 17-times smaller than that for NH_2OH (86 H_2S/PS II versus 5 NH_2OH/PS II). Both H_2S and NH_2OH induce the photoreversible loss of the S_2 state by a reaction that involves an undetermined number of molecules, although the latter is less than or equal to 4 NH_2OH/PS II [7,19]. Like NH_2OH [1,4,6,7,19,20], binding occurs in the dark (S_1 State) at 277 K and is accompanied by two-electron reduction of either the S_1 state in the dark or the S_2 state, following illumination at 200 K. The present data alone do not allow an unambiguous choice between these two options.

The reaction center does indeed turn over during illumination, as seen by the undiminished yield of the reduced primary quinone EPR signal (data not shown). The reversible loss of the S_2 multiline signal is not due to conversion to the $g = 4.1$ form of the manganese complex, as seen by the lack of formation of this signal during the course of the H_2S titration (Fig. 1, curve 4).

From the data in Figs. 1 and 2, we conclude that the mechanism of inhibition by H_2S is qualitatively the same as that expressed by NH_2OH. The mechanism of the latter involves initial binding with the water-oxidizing complex in the S_1 state, followed by two-electron reduction of the manganese complex in either the S_1 or S_2 states. The cumulative evidence from both EPR and EXAFS studies identifies the S_2 state as the target for reduction initiated either by a flash at room temperature [7] or by CW illumination at 200 K [20]. This is also supported by the faster reactivity between NH_2OH and the S_2 state compared to the slower rate of binding in the dark to the S_1 state [21,7]. The following general scheme summarizes the reactions which this class of inhibitors appear to initiate:

Binding:

$$n\mathrm{I} + \mathrm{PS\ II}(S_1) \rightarrow \mathrm{I}_n \cdot \mathrm{PS\ II}(S_1^*) \qquad (1)$$

S_2 Inactivation:

$$\mathrm{I}_n \cdot \mathrm{PS\ II}(S_1^*) \xrightarrow{h\nu} \mathrm{I}_n \cdot \mathrm{PS\ II}(S_2^*) \rightarrow \mathrm{PS\ II}(S_0^*) + n\mathrm{I}^{ox} \qquad (2)$$

Mn Release:

$$m\mathrm{I} + \mathrm{I}_n \cdot \mathrm{PS\ II}(S_1^*) \rightarrow \mathrm{I}_n \cdot \mathrm{PS\ II}(S_1^*) \cdot \mathrm{I}_m$$

$$\rightarrow 3\mathrm{Mn}^{2+} + \mathrm{PS\ II}(\mathrm{Mn}^*) \qquad (3)$$

Initially, the inhibitor (I) binds in the dark S_1 state to a site on the water-oxidizing complex designated PS II(S_1) at a stoichiometry of nI/PS II, $n \leqslant 4$ (reaction 1). Upon illumination, formation of a stable S_2 state is suppressed by facile two-electron reduction to form S_0^* and oxidized I (reaction 2). In the dark, an excess of the inhibitor can bind to initiate the release of 3Mn/PS II and to irreversibly eliminate O_2 evolution (reaction 3). This model disagrees with the recent model given by Beck and Brudvig [6] for NH_2OH inhibition, which proposes direct reduction of S_1 in the dark instead of S_2 reduction. This is based on the observation that once NH_2OH binds to the site on the water-oxidizing complex, it is not in equilibrium with free NH_2OH, which can be scavenged with chemical oxidants. This could be attributed to strong binding in the S_1 state or to a lack of accessibility of the scavengers to the NH_2OH site, rather than reduction of S_1.

Radmer and Ollinger [22,23] have given evidence showing that N- and O-substituted derivatives of NH_2OH are less effective inhibitors of O_2 evolution, in a manner which correlates with their size rather than redox potential. Since H_2S is smaller than NH_2OH, size alone cannot be a criterion for the weaker inhibition constant of H_2S at the water-oxidizing complex site. This is consistent with earlier work on NH_2OH emphasizing the importance of local environmental effects rather than inhibitor size alone as the determining factor in inhibitor effectiveness [12].

Two possible reasons for the weaker inhibition constant of H_2S versus NH_2OH could be a lower redox potential for oxidation, or a lower binding affinity around the site for oxidation. We will consider the latter case first, and will use divalent ion stability constant data from the literature for comparison [24]. This is a valid comparison only if the binding sites for H_2S and NH_2OH are the same and if it involves a metal ion with a net charge of +2. Although there is circumstantial information supporting binding of these analogs

to manganese, there is little direct information. Furthermore, although there is good evidence that the oxidation state of manganese is Mn(III) in the S_1 state [25,26], the net charge is unknown. If binding affinity alone was the source of the difference in inhibition, then we would expect just the opposite result, since S donor ligands bind with greater affinity to divalent aqueous metal ions than do N or O donor ligands of comparable size. This may be seen in the association constants for divalent metal ions in aqueous solution with the chelating ligand $XCH_2CH_2NH_2$ given in Eqn. (4) [24].

$$M^{2+} + XCH_2CH_2NH_2 \rightarrow M(XCH_2CH_2NH_2)^{2+} \quad (4)$$

For M = Zn and X = OH, NH_2 and SH, log K = 3.7, 5.7 and 10.22, respectively. For M = Ni and X = OH, NH_2 and SH, log K = 2.98, 7.35 and 10.05, respectively. These data suggest that the relative binding affinities alone for S versus N and O donor ligands do not account for the difference in inhibition of H_2S versus NH_2OH at the water-oxidizing site. Hence, there must be other factors that are important, such as binding to a site other than Mn, or reversed binding affinities for higher oxidation states than Mn^{2+}, or an unusual local environment around the manganese, or different oxidation potentials for NH_2OH versus H_2S. The preference for coordination of smaller atomic radii ligands with increasing oxidation state of manganese (MnO_2, Mn_2O_3, MnO_4^-, for example) in synthetic compounds may also account for the higher reactivity of NH_2OH over H_2S with the water-oxidizing complex.

We next consider differences in the redox properties of these inhibitors. Both NH_2OH and H_2S can undergo two-electron oxidation chemistry. The thermodynamically most favorable reactions to consider in aqueous solutions are listed in Eqns. 5, 6 and 7

	Redox potential	
$2NH_2OH \leftrightarrow 2e^- + 2H_2O + 2H^+ + N_2(g)$	+1.69 V	(5)
$HS^- + OH^- \leftrightarrow 2e^- + H_2O + S_{(s)}$	+0.52 V	(6)
$H_2S_{(aq)} \leftrightarrow 2e^- + 2H^+ + S_{(s)}$	+0.14 V	(7)

The acid dissociation constants for NH_3OH^+ and H_2S are $1.1 \cdot 10^{-6}$ [27] and $1.0 \cdot 10^{-7}$ [28], respectively, from which we see that there is a considerable amount of the conjugate bases for both these weak acids in solution at the pH of our experiments (pH 6.5). Only the neutral form of NH_2OH has been found to be effective for inhibition of water oxidation [2]. In the case of H_2S, the pH dependence has not been examined. The data in Eqns. 5–7 indicate that NH_2OH is a considerably stronger two-electron reductant than H_2S or HS^- in aqueous media. While there is evidence pointing to N_2 as the only gaseous product of the reaction between PS II and NH_2OH [22,23], no information is yet available on the products of the reaction with H_2S. The site of formation of N_2 is still controversial, with evidence pointing to the water-oxidizing complex [22,23] and to the one-electron donor Z^+ [6]. If the thermodynamic differences in eqns. 5 and 6 were the sole determining factor in the inhibition constants, then one could expect that NH_2H would exhibit a much greater inhibition constant by a factor of 10^{40}. Such a difference is not observed, so these simple arguments are unable to quantitatively account for the factor of 17 difference in inhibition expressed by the these substrate analogs, although the relative order is correctly predicted. Either environmental factors in the binding site could be important in reducing the difference between these potentials, or the reaction with the water-oxidizing complex which causes inhibition of the S_2 state may involve reactions other than those considered in Eqns. 5–7.

The irreversible reaction of S_1 with H_2S

Fig. 4 gives a plot of data taken from Figs. 2 and 1 (curve 3), graphed as the number of Mn which remain bound vs. the % O_2 evolution activity. The data were fitted to a straight line using a least-squares fit (residual = 0.93). Because the slope is constant, it shows that, throughout the course of the titration, all PS II centers release the same number of Mn ions. The value of the slope gives the average number of Mn ions that are released per inactivation event (2.7 ± 0.3 Mn/PS II, the error is set by the limits on the number of Chl per PS II). Also shown in Fig. 4 is equivalent titration curve for NH_2OH; the raw data were taken from Ref. 7. This also shows a linear relationship with the same slope (residual = 0.99), demonstrating that the manganese site behaves

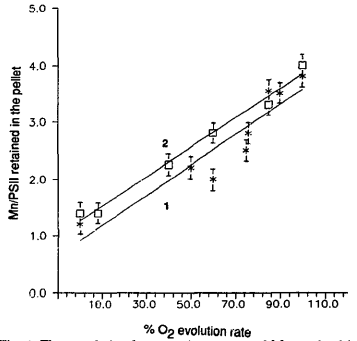

Fig. 4. The correlation between the amount of Mn retained in the PS II complex as a function of the % of O_2 evolution activity remaining following treatment with H_2S (curve 1) or NH_2OH (curve 2). The data for H_2S are from Figs. 1 and 2, while the NH_2OH data are from Ref. 7.

the same way with both inhibitors. These data suggest that both H_2S and NH_2OH react with the water-oxidizing complex to release manganese cooperatively in amounts equal to about 2.7 Mn/PS II. This would come about most easily if there is a common binding site for at least 3 Mn ions, either as a single cluster or perhaps in association with the remaining 1 Mn/PS II which is not as easily extracted. This is consistent with earlier EPR studies [25,29,30] and EXAFS studies [26,31], establishing the existence of a cluster of 2–4 Mn ions.

Reactivity of H_2S with signal II

Fig. 3 shows that signal II is reduced by H_2S in a reaction that occurs in parallel with the release of Mn. This indicates that accessibility to the radical formed from ^{160}Tyr located on the D_2 polypeptide, which is believed to be responsible for signal II [32], is controlled by the release of Mn. This conclusion is in agreement with the enhanced decay kinetics observed previously for the reduction of signal II by exogenous donors in Mn-depleted membranes [33].

Lack of H_2S binding to the primary electron acceptor

There is at least one difference in the mode of reactivity of H_2S vs. NH_2OH. Unlike NH_2OH, H_2S does not have a high affinity binding site affecting the Q_A-His-Fe primary electron acceptor complex of PS II, as seen by the lack of any change in the EPR signals for the photo-reduced semiquinone. If binding of H_2S at the acceptor site occurs, it must have an affinity which is more than 100-fold weaker than that of NH_2OH. The effect of NH_2OH binding to this site is to induce a large shift of the normal EPR signal observed at $g = 1.9–2.1$ [7,11]. The lack of a shift with H_2S may not be understandable in terms of differences in binding affinity to the ferrous ion, since sulfur donor ligands bind considerably more strongly (10^6–10^7) than oxygen and nitrogen donor ligands to coordinately unsaturated divalent transition metal ions (vide supra). This situation may be reversed if the metal ion already has several strongly bound ligands, such as the four imidazole ligands presumed to coordinate to the ferrous ion by analogy with bacterial reaction centers [11]. In such cases, a weaker π donor ligand than H_2S, such as NH_2OH, may exhibit stronger binding to the metal. Other factors, such as relative strengths of H-bonding, may play a more important role in determining binding affinities of these inhibitors to the acceptor site.

Acknowledgements

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