

Topography of the O₂-evolving site determined with water analogs

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Photosystem II

Oxygen evolution

Spinach

Hydroxylamine derivative

Chloroplast

Hydrazine derivative

1. INTRODUCTION

Hydroxylamine (NH₂OH) and hydrazine (NH₂NH₂) are both analogs of two molecules of H₂O. As such, they appear to be competitive inhibitors of photosystem II-mediated H₂O oxidation: at low concentrations they are able to override H₂O oxidation without destroying the O₂-evolving system.

We have previously shown [1] that incubation of chloroplasts with low concentrations of NH₂OH in the dark results in the reduction of S₁ to S₀ and the subsequent binding of one molecule of NH₂OH. According to these results, the two-flash delay in O₂ evolution [2] at low NH₂OH concentrations reflects the oxidation of one molecule of NH₂OH, after which O₂ evolution proceeds normally starting from S₀ [1].

Here, we describe experiments in which we compared the 'donor activities' of various substituted NH₂OH and NH₂NH₂ compounds with the ability of these same donors to interact with the intact O₂-evolving system. The ability of the compound to act as a donor was determined by monitoring fluorescence rise curves in Tris-extracted chloroplasts as a function of donor concentration. Interaction with the O₂-evolving system was determined from the concentration dependence of the delay in the maximum O₂ flash yield.

Our results showed that the ability of a given donor to interact with the intact O₂-evolving system correlates with the shape of the molecule rather than its donor activity, and suggest that the

two H₂O-binding sites (which are ~1.47 Å apart) [1] reside in a cleft ~4 Å wide and ~2.5 Å deep. Observed differences between extracted and O₂-competent chloroplasts with different donors suggest that either:

- (1) Tris extraction removes or alters the protein forming the cleft; or
- (2) these donors react other than at the O₂-evolving site in extracted chloroplasts.

2. METHODS

A mass spectrometric apparatus and measuring technique similar to those in [1] and [3] were used to monitor O₂ flash yields. Fluorescence rise curves were obtained as in [4]. Experiments were performed with spinach chloroplasts [5,6] extracted with a high concentration of Tris [5,7] when desired. Sources of the substrates were: hydroxylamine HCl, hydroxylamine-*O*-sulfonic acid, *O*-methylhydroxylamine HCl, *N*-methylhydroxylamine HCl, methylhydrazine sulfate, and *N,N'*-dimethylhydrazine diHCl (sym), from ICN Pharmaceuticals (Plainview, NY), hydrazine sulfate, from Fisher (Fair Lawn, NJ), *O,N*-dimethylhydroxylamine HCl, from Aldrich (Milwaukee, WI) and 1,1-dimethylhydrazine, from Eastman (Rochester, NY).

3. RESULTS AND DISCUSSION

3.1. Donor activity of NH₂OH and NH₂NH₂ derivatives

Table 1 presents a summary of results obtained

Table 1

Concentration of donor at which the fluorescence rise is half-maximal

Compound	Half-maximal conc. (mM)
CH ₃ NHOH	0.1
(CH ₃) ₂ NNH ₂	0.3
NH ₂ NHCH ₃	0.5
NH ₂ OH	0.6
CH ₃ HNNHCH ₃	0.6
NH ₂ NH ₂	0.7
NH ₂ OSO ₃ H	1
CH ₃ HNOCH ₃	3
NH ₂ OCH ₃	4

The reaction mixture for each expt contained 50 mM Tricine (pH 7.4), 5 mM MgCl₂, and Tris-extracted chloroplasts (6 µg chl/ml), in addition to the donor compound. Fluorescence rise curves were obtained after 5 min dark time. Donor-mediated live fluorescence was computed from the expression

$$F(\text{conc.}x) - F(\text{no donor})$$

$$F(\text{saturating conc.}) - F(\text{no donor})$$

where F is the maximum fluorescence yield. Light intensity, computed from the half-time of the rise curve in the presence of DCMU, was $\sim 40 \text{ hits trap}^{-1} \text{s}^{-1}$

when NH₂OH and NH₂NH₂ derivatives were tested for their ability to mediate the fluorescence rise curve in Tris-extracted chloroplasts. Each entry of the table represents the donor concentration at which the fluorescence rise is half-maximal under our assay conditions. (The level of the fluorescence rise reflects the balance between the rate of electron input to photosystem II and the withdrawal from photosystem I via the Mehler reaction.) The compounds are ordered from high to low affinity, which we will take as a measure of their relative donor abilities. These data correlate well with the relative reactivities of the compounds as general electron donors [8,9].

The data of table 1 suggest that:

- The monomethyl- and the two dimethyl-substituted hydrazines can serve as electron donors at least as well as the unsubstituted compound.
- N*-methyl hydroxylamine is at least as good a donor as the parent compound.

- O*-methyl-substituted hydroxylamines generally are poor donors.

3.2. Interaction of NH₂OH and NH₂NH₂ derivatives with the intact O₂-evolving system

Fig.1 shows the concentration dependence of the fifth O₂ flash yield (Y_5) as a function of NH₂OH and its *N*-methyl derivative. The increase of Y_5 is a sensitive indicator of the non-destructive interaction of NH₂OH and NH₂NH₂ with the intact O₂-evolving system [2,10]. The decrease at higher concentrations reflects the parallel and competing destruction of the O₂-evolving system [11]. Note the striking difference between the two donors with respect to their ability to increase Y_5 , suggesting that *N*-methyl substitution greatly decreases the ability of the donor to interact with the intact O₂-evolving system. The difference in the amplitude of the maxima probably reflects the dissimilar interaction-ability/destruction-ability ratios of the two compounds.

Table 2 is a compilation of a series of experiments like those illustrated by fig.1; the concentrations of various NH₂OH and NH₂NH₂ derivatives at which Y_5 is maximal are presented. Note that interaction ability (column 1, table 2)

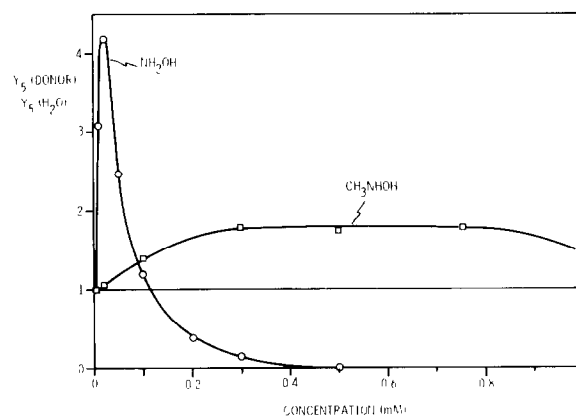


Fig.1. Fifth flash yield of O₂-evolution (Y_5) normalized to Y_5 in the absence of donor, as a function of NH₂OH and CH₃NHOH concentration. The reaction medium contained ¹⁸O₂-equilibrated Tricine (50 mM, pH 7.4), 1 mM MgCl₂, and the appropriate donor concentration. Ten µl of chloroplasts (6 mg chl/ml) were used for each measurement. Flash spacing was 3 s. A 10-min dark period preceded each measurement.

Table 2

Concentration of donor at which Y_5 is maximal

Compound	Concentration (mM)	Normalized value
NH ₂ OH (95%)	0.02	0.03
NH ₂ OSO ₃ H (97%)	0.03	0.03
NH ₂ NH ₂ (>99%)	0.05	0.07
NH ₂ OCH ₃ (97%)	0.25	0.06
NH ₂ NHCH ₃ (97%)	0.27	0.54
CH ₃ NHOH (98%)	0.5	5
CH ₃ HNNHCH ₃ (99%)	20	33
(CH ₃) ₂ NNH ₂ (>98%)	^a	—
CH ₃ HNOCH ₃ (98%)	^a	—

^a No discernible increase in Y_5 up to 100 mM

Data in the first column were obtained from expts like those used to generate fig.1. Normalized values were obtained by dividing these values by those of table 1. Minimum assay values in parentheses were obtained from the respective suppliers

does not correlate with relative (chemical) donor ability (table 1). Even more striking differences appear when these data are adjusted for their differences in donor activity (column 2).

These data suggest that substituting a -CH₃ for an -H, particularly on a nitrogen, greatly decreases the ability of the compound to interact with the intact O₂-evolving system. It appears that the shape of the molecule, rather than its chemical reactivity, is the primary determinant for interaction with the O₂ site.

3.3. Proposed model

The data of sections 3.1 and 3.2 show that:

- (1) The ability of a given donor to interact with the intact O₂-evolving system is not closely related to its donor activity;
- (2) Interaction ability does correlate with the shape of the molecule. More specifically, only unsubstituted or *O*-substituted compounds are able to interact readily with the intact O₂-evolving system. *N*- and (particularly) di-*N*-substitution greatly diminishes interaction, which probably correlates with the greater width of *N*-substituted vs *O*-substituted (or unsubstituted) compounds.

Fig.2 is an illustration of our concept of the O₂-evolving site. It has two main features:

- (1) There are two binding sites ~1.47 Å apart [1]; and
- (2) These binding sites reside in a cleft, the shape of which is reflected in the relative accessibility of the H₂O analogs.

Four examples are presented:

- (1) NH₂NH₂, a direct (unsubstituted) analog of two molecules of H₂O (2H₂O would look about the same);
 - (2) NH₂OSO₃H, a bulky, highly active compound;
 - (3) CH₃HNNH₂, a relatively small, poorly active compound;
 - (4) (CH₃)₂NNH₂, a bulky, non-active compound.
- Note that with NH₂NH₂ and NH₂OSO₃H the unbonded electron pairs of N and O can easily interact with the positive binding sites of the O₂ system. CH₃HNNH₂ can fit in the cleft, but only in a twisted configuration that prohibits an effective interaction. This may correlate with the intermediate position of this donor in the hierarchy of table 2. (CH₃)₂NNH₂ does not fit in the cleft, and does not interact with the intact O₂ system (cf. table 2). The same results hold true for the molecules not pictured. The first 4 compounds of table 2, which readily interact with the intact O₂ system, fit into the cleft and can interact with the binding sites. The last 5 compounds neither readily interact with the O₂ system nor correctly fit into the cleft.

We conclude that the H₂O binding sites lie in a cleft ~4 Å wide and ~2.5 Å deep. This topography provides a means by which the light-generated oxidant can be protected in the chloroplast, and may explain why the O₂-evolving system seems to be inaccessible to most common redox mediators [12]. In Tris-extracted chloroplasts there is apparently no such topographical barrier, suggesting that either (1) artificial donors in extracted chloroplasts are oxidized at a site other than the inactivated O₂-evolving site, or (2) Tris-extraction and the consequent depletion of Mn result in the loss of the cleft. The latter supposition is supported by the reported inhibitory effect of glutaraldehyde fixation on the Tris-mediated inactivation of O₂-evolution [13,14], and the reported loss and reconstitution of O₂ evolution attributable to Tris-extractable polypeptide of M_r 23 000 [15].

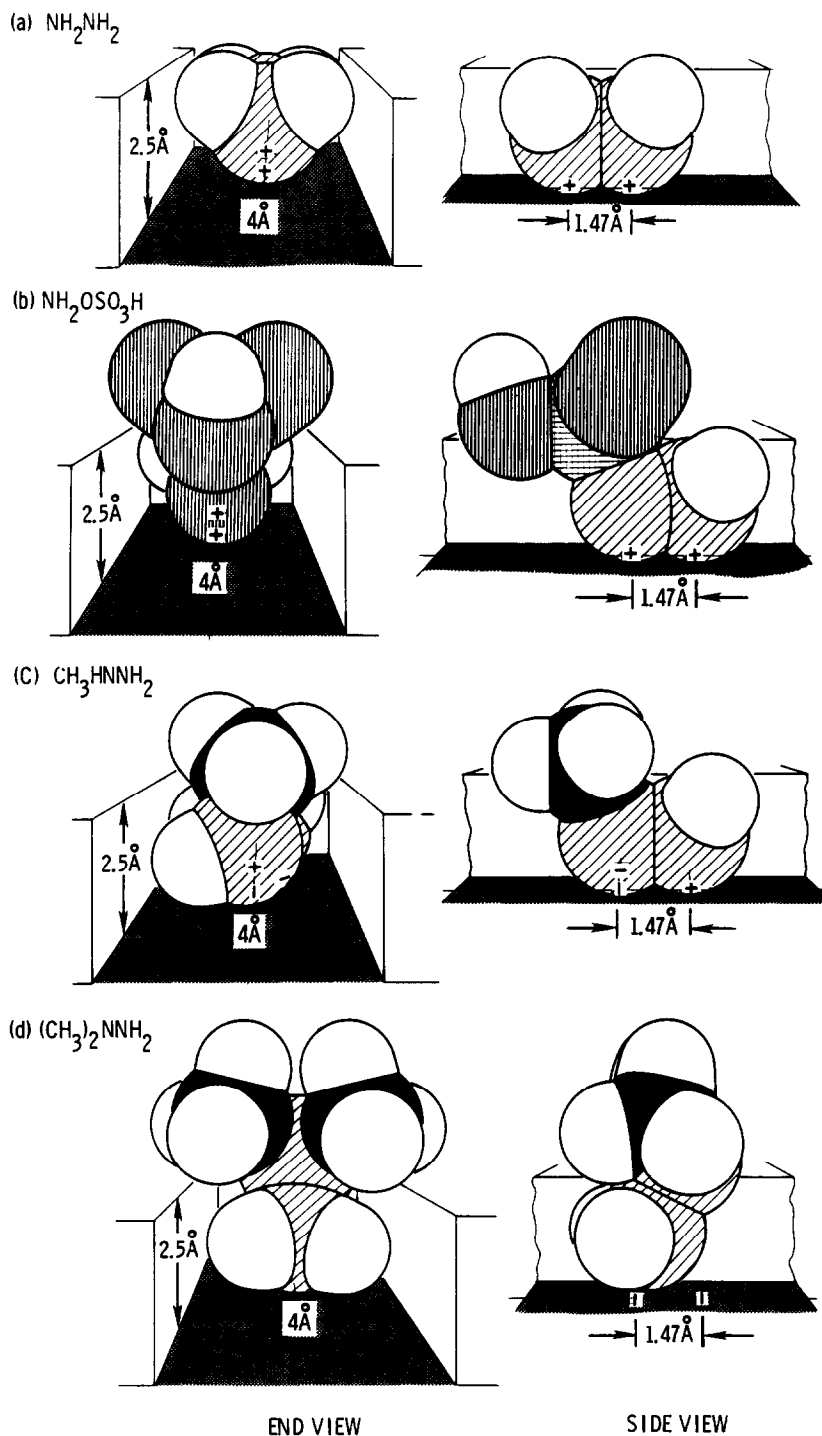


Fig.2. Model of the O_2 -evolving site, and its interaction with 4 H_2O analogs: (1) NH_2NH_2 ; (2) $\text{NH}_2\text{OSO}_3\text{H}$; (3) CH_3HNNH_2 ; (4) $(\text{CH}_3)_2\text{NNH}_2$. The drawings and measurements were made using atom models according to Stuart and Briegleb (Arthur LaPine, Chicago, IL). Atoms are coded as follows: diagonal hatch, N; vertical hatch, O; squares, S; black, C; white, H. The binding site of the O_2 system is denoted by (I), and the unbonded electron pair by (—).

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