Topography of the O_2-evolving site determined with water analogs

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1. INTRODUCTION

Hydroxylamine (NH_2OH) and hydrazine (NH_2NH_2) are both analogs of two molecules of H_2O. As such, they appear to be competitive inhibitors of photosystem II-mediated H_2O oxidation: at low concentrations they are able to override H_2O oxidation without destroying the O_2-evolving system.

We have previously shown [1] that incubation of chloroplasts with low concentrations of NH_2OH in the dark results in the reduction of S_1 to S_0 and the subsequent binding of one molecule of NH_2OH. According to these results, the two-flash delay in O_2 evolution [2] at low NH_2OH concentrations reflects the oxidation of one molecule of NH_2OH, after which O_2 evolution proceeds normally starting from S_0 [1].

Here, we describe experiments in which we compared the 'donor activities' of various substituted NH_2OH and NH_2NH_2 compounds with the ability of these same donors to interact with the intact O_2-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 O_2-evolving system was determined from the concentration dependence of the delay in the maximum O_2 flash yield.

Our results showed that the ability of a given donor to interact with the intact O_2-evolving system correlates with the shape of the molecule rather than its donor activity, and suggest that the two H_2O-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 O_2-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 O_2-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 O_2 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 NH_2OH and NH_2NH_2 derivatives*

Table 1 presents a summary of results obtained

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Table 1

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

Compound	Half-maximal conc. (mM)
CH_3NHOH	0.1
$(CH_3)_2NNH_2$	0.3
NH_2NHCH_3	0.5
NH_2OH	0.6
$CH_3HNNHCH_3$	0.6
NH_2NH_2	0.7
NH_2OSO_3H	1
CH_3HNOCH_3	3
NH_2OCH_3	4

The reaction mixture for each expt contained 50 mM Tricine (pH 7.4), 5 mM $MgCl_2$, 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

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

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

when NH_2OH and NH_2NH_2 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:

(i) The monomethyl- and the two dimethyl-substituted hydrazines can serve as electron donors at least as well as the unsubstituted compound.

(ii) *N*-methyl hydroxylamine is at least as good a donor as the parent compound.

(iii) *O*-methyl-substituted hydroxylamines generally are poor donors.

3.2. *Interaction of NH_2OH and NH_2NH_2 derivatives with the intact O_2-evolving system*

Fig.1 shows the concentration dependence of the fifth O_2 flash yield (Y_5) as a function of NH_2OH and its *N*-methyl derivative. The increase of Y_5 is a sensitive indicator of the non-destructive interaction of NH_2OH and NH_2NH_2 with the intact O_2-evolving system [2,10]. The decrease at higher concentrations reflects the parallel and competing destruction of the O_2-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 O_2-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 NH_2OH and NH_2NH_2 derivatives at which Y_5 is maximal are presented. Note that interaction ability (column 1, table 2)

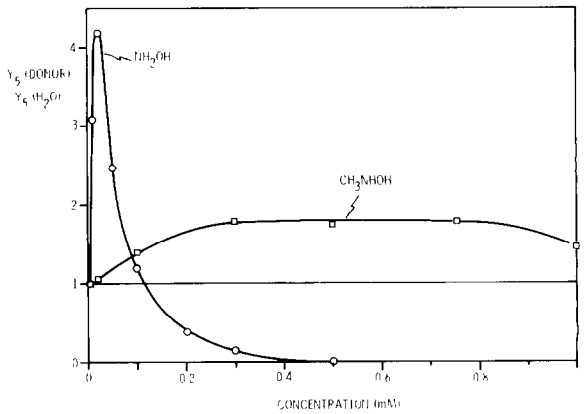

Fig.1. Fifth flash yield of O_2-evolution (Y_5) normalized to Y_5 in the absence of donor, as a function of NH_2OH and CH_3NHOH concentration. The reaction medium contained $^{18}O_2$-equilibrated Tricine (50 mM, pH 7.4), 1 mM $MgCl_2$, and the appropriate donor concentration. Ten μl of chloroplasts (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_2OH (95%)	0.02	0.03
NH_2OSO_3H (97%)	0.03	0.03
NH_2NH_2 (>99%)	0.05	0.07
NH_2OCH_3 (97%)	0.25	0.06
NH_2NHCH_3 (97%)	0.27	0.54
CH_3NHOH (98%)	0.5	5
$CH_3HNNHCH_3$ (99%)	20	33
$(CH_3)_2NNH_2$ (>98%)	[a]	–
CH_3HNOCH_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 $-CH_3$ for an -H, particularly on a nitrogen, greatly decreases the ability of the compound to interact with the intact O_2-evolving system. It appears that the shape of the molecule, rather than its chemical reactivity, is the primary determinant for interaction with the O_2 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 O_2-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 O_2-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 O_2-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 H_2O analogs.

Four examples are presented:

(1) NH_2NH_2, a direct (unsubstituted) analog of two molecules of H_2O ($2H_2O$ would look about the same);

(2) NH_2OSO_3H, a bulky, highly active compound;

(3) CH_3HNNH_2, a relatively small, poorly active compound;

(4) $(CH_3)_2NNH_2$, a bulky, non-active compound.

Note that with NH_2NH_2 and NH_2OSO_3H the unbonded electron pairs of N and O can easily interact with the positive binding sites of the O_2 system. CH_3HNNH_2 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. $(CH_3)_2NNH_2$ does not fit in the cleft, and does not interact with the intact O_2 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 O_2 system, fit into the cleft and can interact with the binding sites. The last 5 compounds neither readily interact with the O_2 system nor correctly fit into the cleft.

We conclude that the H_2O 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 O_2-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 O_2-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 O_2-evolution [13,14], and the reported loss and reconstitution of O_2 evolution attributable to Tris-extractable polypeptide of M_r 23000 [15].

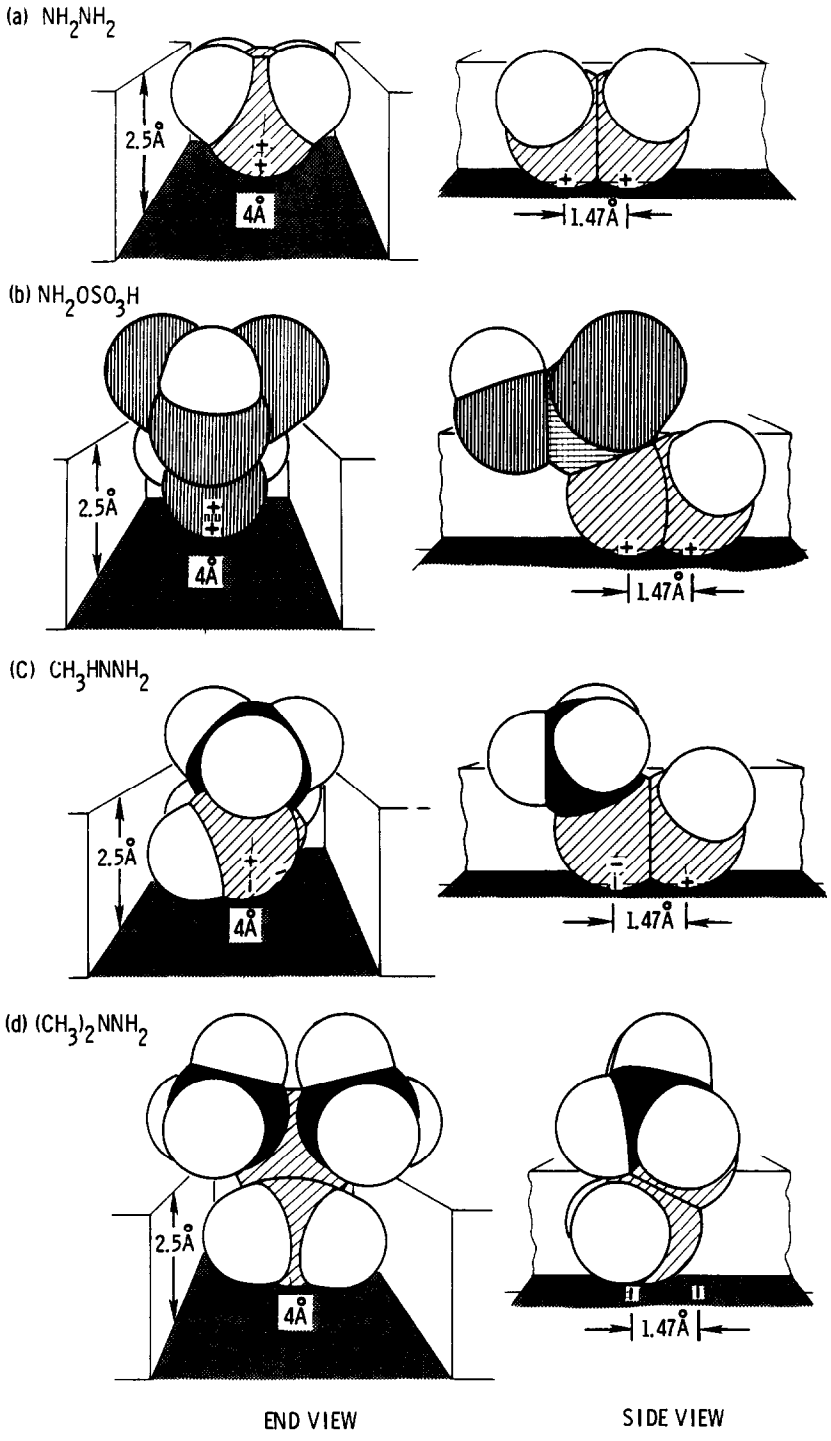

Fig.2. Model of the O_2-evolving site, and its interaction with 4 H_2O analogs: (1) NH_2NH_2; (2) NH_2OSO_3H; (3) CH_3HNNH_2; (4) $(CH_3)_2NNH_2$. The drawings and measurements were made using atom models according to Stuart and Briegleb (Arthur LaPine, Chicago, IL). Atoms are coded as follows: diagonal hatch, N; vertical hatch, O; squares, S; black, C; white, H. The binding site of the O_2 system is denoted by (|), and the unbonded electron pair by (—).

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