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Characterization of inhibitory effects of NH_2OH and its N-methyl derivatives on the O_2-evolving complex of Photosystem II

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Abstract

Inorganic cofactors (Mn, Ca^{2+} and Cl^-) are essential for oxidation of H_2O to O_2 by Photosystem II. The Mn reductants NH_2OH and its N-methyl derivatives have been employed as probes to further examine the interactions between these species and Mn at the active site of H_2O oxidation. Results of these studies show that the size of a hydroxylamine derivative regulates its ability to inactivate O_2 evolution activity, and that this size-dependent inhibition behavior arises from the protein structure of Photosystem II. A set of anions (Cl^-, F^- and SO_4^{2-}) is able to slow NH_2OH and CH_3NHOH inactivation of intact Photosystem II membranes by exerting a stabilizing influence on the extrinsic 23 and 17 kDa polypeptides. In contrast to this non-specific anion effect, only Cl^- is capable of attenuating CH_3NHOH and $(CH_3)_2NOH$ inhibition in salt-washed preparations lacking the 23 and 17 kDa polypeptides. However, Cl^- fails to protect against NH_2OH inhibition in salt-washed membranes. These results indicate that the attack by NH_2OH and its N-methyl derivatives on Mn occurs at different sites in the O_2-evolving complex. The small reductant NH_2OH acts at a Cl^--insensitive site whereas the inhibitions by CH_3NHOH and $(CH_3)_2NOH$ involve a site that is Cl^- sensitive. These findings are consistent with earlier studies showing that the size of primary amines controls the Cl^- sensitivity of their binding to Mn in the O_2-evolving complex.

Abbreviations: MES – 4-morpholinoethanesulfonic acid; PS II – Photosystem II

Introduction

The pioneering observations of Joliot and Kok (1975) showed that photosynthetic water oxidation occurs by successive formation of five oxidation states, S_i (i = 0 to 4); S_4 decays spontaneously to produce S_0 and O_2. The S-state transitions appear to correspond to oxidation state changes in the 4 Mn atoms associated with the O_2 evolving complex of Photosystem II (PS II) (Babcock 1987). In addition to Mn, both Ca^{2+} and Cl^- are required for efficient operation of the S-state cycle (Debus 1992), but the precise functions and ligation sites of these cofactors remain uncertain. Hydroxylamine and its derivatives inhibit O_2 evolution activity by reducing Mn from higher oxidation states to Mn^{2+} (Cheniae and Martin 1971), which is subsequently released from PS II (Yocum et al. 1981). The fact that illumination counters this inhibitory action (Sharp and Yocum 1981) may reflect the lowered reactivity of NH_2OH, and NH_2NH_2 as well, with the S_3 state (Messinger et al. 1991). The inhibitory action of NH_2OH on dark-adapted, salt-washed PS II preparations can be slowed by addition of Ca^{2+}, whose action is to stabilize reduced Mn at or near its binding site (Mei and Yocum 1991). Large reductants such as hydroquinone (Ghanotakis et al. 1984), p-phenylenediamine (Mei and Yocum 1992b) and N,N,N',N'-tetramethyl-p-phenylenediamine (Tamura et al. 1986) inhibit O_2 evolution only in PS II membranes

depleted of the extrinsic 23 and 17 kDa proteins. Both Ca^{2+} and Cl^- are able to counter the inhibition induced by such reductants (Mei and Yocum 1992a,b).

We have further characterized the action of reductants on PS II Mn by examining the effect of anions on inactivations of the O_2-evolving complex produced by NH_2OH and its N-methyl derivatives. The results show that the size of hydroxylamines regulates their ability to interact with PS II Mn in intact PS II membranes. A specific effect of Cl^- on CH_3NHOH and $(CH_3)_2NOH$ inhibitions of the O_2-evolving complex in salt-washed PS II membranes demonstrates that the addition of a single methyl group to NH_2OH modifies the inhibitory process by redirecting the inhibitory action of the reductant to a different site within the O_2-evolving complex.

Materials and methods

Photosystem II preparations were isolated using a modification of the method of Berthold et al. (1981). Salt-washed PS II membranes were obtained by exposing PS II preparations to 2 M NaCl for 30 min (dark, 4 °C) at 1.5 mg Chl/ml and a single centrifugation/wash step in MES buffer (pH 6.0). For reductant treatment, intact and salt-washed PS II preparations (2 mg Chl/ml) were incubated with reductants at 4 °C in darkness for increasing periods of time as indicated in Figure and Table legends. At the pH (6.0) used for the incubations, the hydroxylamines exist in solution as an approximately equimolar mixture of the protonated and free base species. Oxygen evolution was assayed in a Clark-type O_2 electrode in 10 mM $CaCl_2$ and 50 mM MES using 0.31 mM 2,6-dichloro-p-benzoquinone as the acceptor.

Results

Work by Radmer and Ollinger (1983) has established the effect of size on activity of hydroxylamines as reductants that interact with the O_2-evolving complex; N-methylation of NH_2OH increases the size of the molecule with only minor changes in other properties of the molecule such as pK_a, whereas O-substitution decreases reactivity towards the S-states. Beck and Brudvig (1988) exposed intact PS II membranes to N-methylated derivatives of NH_2OH and concluded that the S_{-1} state was formed in this system in a reaction that was slowed by the presence of Cl^-. We have extended this investigation so as to examine the ability of NH_2OH and its N-methyl derivatives to inhibit O_2 evolution activity in both intact and salt-washed PS II membranes depleted of extrinsic 23 and 17 kDa proteins. As shown in Fig. 1, the smallest reductant, NH_2OH, is also the most effective inhibitor of the intact O_2-evolving complex. Addition of a methyl group to produce CH_3NHOH diminishes inhibitory capacity relative to NH_2OH, while the largest reductant ($(CH_3)_2NOH$) has no inhibitory effect on intact PS II membranes.

The experiments in Fig. 1 were repeated with salt-washed PS II membranes. Figure 2 shows that after removal of extrinsic 23 and 17 kDa proteins, $(CH_3)_2NOH$ is capable of inactivating O_2 evolution activity; inhibition by 1 mM $(CH_3)_2NOH$ is increased to 70% under conditions where no effect was seen with intact PS II membranes. The extents of inhibition by CH_3NHOH and NH_2OH were also significantly enhanced (Fig. 2). Comparing the data of Figs. 1 and 2 shows that even for hydroxylamines that are small in comparison to large reductants such as hydroquinone or the p-phenylenediamines, presence of the extrinsic 23 and 17 kDa proteins can exert a strong influence on inhibition of the O_2-evolving complex.

We have previously reported that a group of anions (F^-, Cl^-, Br^- and SO_4^{2-}) was capable of slowing NH_2OH inhibition in intact PS II membranes (Mei and Yocum 1990). After removal of the extrinsic 23 and 17 polypeptides, however, these same anions failed to protect against NH_2OH inhibition of activity, suggesting that the anion effect is due to stabilization of binding of the extrinsic 23 and 17 polypeptide during NH_2OH incubation (Mei and Yocum 1990). This same nonspecific anion effect was observed on CH_3NHOH inactivation of intact PS II membranes (data not shown). The effectiveness of anions in interfering with both NH_2OH and CH_3NHOH inhibitions follows the order $F^- > SO_4^{2-} > Cl^- > Br^-$ (data not shown), which is similar to that observed for denaturation or stabilization effects in a number of protein systems (Jencks 1969).

A further investigation was carried out to examine the anion effect on CH_3NHOH and $(CH_3)_2NOH$ inhibition of activity in salt-washed preparations

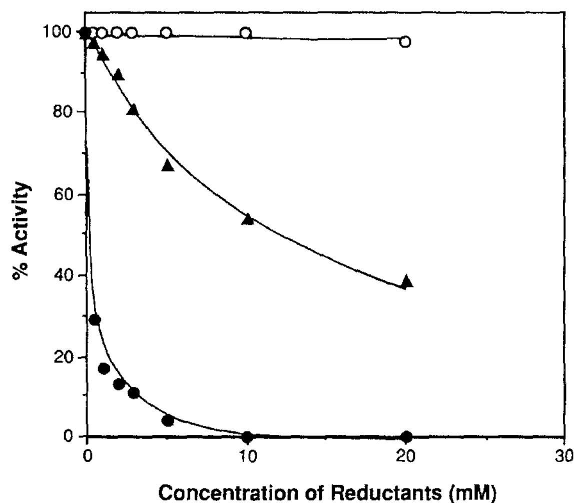

Fig. 1. Comparison of inhibitory effects of NH_2OH and its N-methyl derivatives on intact PS II membranes. Membranes (2 mg Chl/ml) were incubated with the reductants at the concentrations shown for 1 h and diluted 160-fold in assay buffer containing 10 mM $CaCl_2$, 0.31 mM DCBQ and 50 mM MES, pH 6.0. Control (100%) O_2-evolution activity: 650 μmol O_2/mg Chl·h for intact PS II membranes. Open circles: $(CH_3)_2NOH$; triangles: CH_3NHOH; solid circles: NH_2OH.

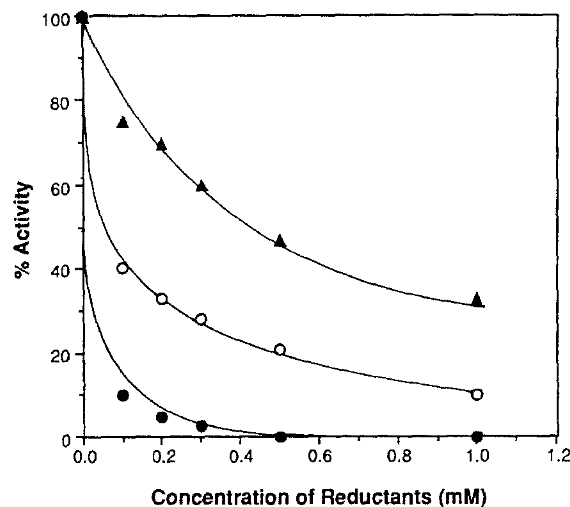

Fig. 2. Comparison of inhibitory effects of NH_2OH and its N-methyl derivatives on salt-washed PS II membranes. The salt-washed PS II membranes (2 mg Chl/ml) were incubated with the reductants at the concentrations shown for 1 h. Assay conditions are as described in Fig. 1. Control (100%) O_2-evolution activity: 400 μmol O_2/mg Chl·h for salt-washed PS II preparations. Triangles: $(CH_3)_2NOH$; open circles: CH_3NHOH; solid circles: NH_2OH.

lacking the 23 and 17 kDa polypeptides. As shown in Fig. 3, addition of Cl^- attenuates inhibition by both N-methyl derivatives of NH_2OH while the anion fails to produce any measurable effect on NH_2OH inhibition. In order to separate Cl^- effects related to interactions between hydroxylamines and Mn from other, nonspecific anion effects described above, SO_4^{2-} and F^- salts were used to replace Cl^- during the reductant exposure step. As shown in Table 1, SO_4^{2-} has no effect on either CH_3NHOH or $(CH_3)_2NOH$ inhibition, and F^- interferes only slightly with the inhibitions. On the basis of data in Table 1, it appears that the interference by Cl^- with CH_3NHOH and $(CH_3)_2NOH$ inhibitions of activity does not arise from lyotropic effects (Homann 1989) on the PS II protein matrix.

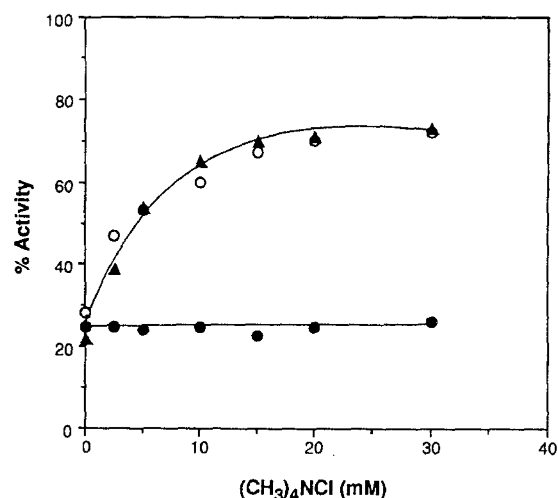

Fig. 3. Effect of Cl^- on inhibition by NH_2OH and its N-methyl derivatives in salt-washed PS II membranes. In the presence of various Cl^- concentrations, the salt-washed PS II membranes (2 mg Chl/ml) were incubated with 0.1 mM NH_2OH for 3 min (solid circles), 0.2 mM CH_3NHOH for 10 min (open circles), and 2 mM $(CH_3)_2NOH$ for 20 min (triangles). Assay conditions and control activity are as described in the legends of Figs. 1 and 2.

Discussion

Comparison of the inhibitory actions of NH_2OH, CH_3NHOH and $(CH_3)_2NOH$ on intact PS II membranes provides further evidence of the extent to which the Mn complex is shielded by the 23 and 17 kDa polypeptides. The increase in size created by addition of a single methyl group to NH_2OH substantially decreases inactivation of the O_2-evolving complex in intact PS II membranes; the presence of a second methyl group abolishes

Table 1. Effect of anions on CH_3NHOH and $(CH_3)_2NOH$ inhibition of O_2 evolution activity in salt-washed PS II membranes

Anion (mM)	% Activity remaining after incubation with:	
	0.2 mM CH_3NHOH	2 mM $(CH_3)_2NOH$
None	30	35
50 mM $(CH_3)_4NCl$	85	87
50 mM $(CH_3)_4NF$	50	51
50 mM $(CH_3)_4NHSO_4$	37	35

The control (100%) activity was 400 μmoles O_2/mg Chl·h for salt-washed membranes. The incubations with CH_3NHOH or $(CH_3)_2NOH$ were 10 and 20 min, respectively. Assay conditions were as described in Fig. 1.

inhibition entirely under the conditions employed in Fig. 1. A further examination of the behavior of CH_3NHOH and $(CH_3)_2NOH$ in salt-washed PS II membranes (Fig. 2) shows that removal of the topological barrier between PS II Mn and the external medium that is provided by the extrinsic 23 and 17 kDa proteins increases exposure of the Mn complex of PS II to reduction and inhibition by both methyl derivatives of NH_2OH. This observation would indicate that the major difference between NH_2OH and its N-methyl derivatives as inhibitors of the O_2-evolving complex is size, which decreases the ability of the N-methyl derivatives to interact with PS II Mn. It should be noted, however, that although the Mn complex in salt-washed membranes is more exposed to exogenously added reagents, the presence of the extrinsic 33 kDa protein might still regulate access of large reductants to the Mn complex. If so, this would account for the remaining size-dependent inhibition pattern for hydroxylamines that is observed in salt-washed PS II membranes (Fig. 2).

We have previously shown that Cl^- has no effect on the kinetics of NH_2OH inhibition in salt-washed PS II preparations (Mei and Yocum 1991). However, the data of Fig. 3 show that under the same conditions Cl^- impedes activity inhibition by CH_3NHOH and $(CH_3)_2NOH$. These observations would suggest either that NH_2OH and its N-methyl derivatives attack Mn at different sites within the O_2-evolving complex or that steric factors produce an environment where only the larger, methylated hydroxylamine species are affected by the presence of Cl^-. In addition, our data are consistent with earlier studies on the effects of size on binding of primary amines to Mn in PS II (Sandusky and Yocum 1984). Thus for amines as well as for redox-active hydroxylamines, the larger reacting species exert their action on the Mn cluster at sites normally occupied by Cl^- or, alternatively, by way of sites within the O_2-evolving complex where the presence of Cl^- can interfere with the inhibition process.

The nature of the relationship between Cl^- and the site of action of hydroxylamines in PS II is addressed in part by our results with other anions. As we show in Table 1, anion-induced retardation of CH_3NHOH and $(CH_3)_2NOH$ inhibition of activity in salt-washed PS II membranes is Cl^- specific; SO_4^{2-} and F^- do not produce the same protective effect. Although SO_4^{2-} is able to stabilize PS II structure (Homann 1988), it is not competitive with Cl^- in the O_2-evolving complex (Sandusky and Yocum 1986). If Cl^- interference with the inhibition of the O_2-evolving complex by N-methylated hydroxylamines were due to its ability to exert a purely lyotropic effect on protein structure and stability similar to the effects observed for SO_4^{2-} in intact PS II preparations (Homann 1989), then the latter anion should also retard inhibitions produced by hydroxylamines in salt-washed PS II membranes. Such an effect is not observed (Table 1). As we also show in Table 1, F^- strongly affects both NH_2OH and CH_3NHOH inhibition of intact PS II membranes but exerts a weaker interference with CH_3NHOH and $(CH_3)_2NOH$ inhibition of salt-washed membranes. This contrasts with results obtained under conditions of illumination, where F^- has been shown to compete with Cl^- for its binding site in PS II (Sandusky and Yocum 1986).

The contrasting properties of anions that we document here with respect to inhibition by hydroxylamines of intact and salt-washed PS II membranes clearly demonstrates the existence of two distinctive anion effects on PS II: 1) nonspecific anion effects that appear to be lyotropic in nature and are exerted directly or indirectly on the extrinsic 23 and 17 kDa proteins during the action of exogenous reductants on the O_2-evolving complex in intact PS II membranes; and 2) a specific Cl^- effect which may directly regulate the interaction between the Mn complex and reductants larger than NH_2OH. Although it is not possible to exclude a Cl^--protein interaction as the origin of the phenomena we report here, the absence of obvious lyotropic effects on inhibition by hydroxylamines suggests that the antagonism between Cl^- and N-methylated hydrox-

ylamines occurs by a mechanism similar to that already demonstrated for binding by primary amines to the O_2-evolving complex.

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