Biochimica et Biophysica Acta 809 (1985) 245–259
Elsevier

BBA 41828

Effects of Photosystem II extrinsic proteins on microstructure of the oxygen-evolving complex and its reactivity to water analogs

Noriaki Tamura and George Cheniae

University of Kentucky, Lexington, KY 40546-0091 (U.S.A.)

(Received May 20th, 1985)

Key words: Photosystem II; Oxygen evolution; Hydroxylamine; Water splitting; Manganese; (Wheat chloroplast)

We used Triton-prepared PS II membranes in studies of the inactivation of O_2 evolution and solubilization of Mn and specific PS II polypeptides by NH_2OH, N- and O-substituted NH_2OH derivatives, NH_2NH_2 and NH_4Cl. The inactivation of O_2-evolution, solubilization of Mn and the solubilization of the extrinsic PS II polypeptides (17, 23 and 33 kDa) proved closely correlated, half-maximal effects occurring with only 100 μM NH_2OH. NH_2OH (2 mM) and NaCl (1 M) extractions solubilized about one-half the amount of protein solubilized by 0.8 M Tris-HCl (pH 8.0). The inactivation of the Mn-S-state complex proceeded by apparent first-order kinetics, the rate constant dependent on NH_2OH (CH_3NHOH) concentration and pH. In the range of micromolar concentrations of NH_2OH, this inactivation did not occur via a cooperative type mechanism. Depletion of the 17 and 23 kDa proteins modified the pH dependency of inactivation (from pH 7.8 to 6.5) and also resulted in an approx. 2-fold maximum increase in the inactivation rate constant. Significantly, reconstitution of such NaCl-TMF-2 membranes with the 17 and 23 kDa proteins reverted both the pH dependency and the inactivation rate constant to that of TMF-2. A hierarchy of effectivity for solubilization of Mn and protein, which was highly correlated with inactivation of the Mn-S-state enzyme, was observed among NH_2OH and its derivatives. This same hierarchy was observed irrespective of prior depletion of the 17 and 23 or the 17, 23 and 33 kDa proteins from TMF-2. The hierarchy of effectivity among derivatives was: $NH_2OH > CH_3NHOH > NH_2NH_2$, $NH_2OSO_3 > NH_2OCH_3 \gg CH_3NHOCH_3$, NH_4Cl. The function(s) of the extrinsic PS II proteins as determinants of the reactivity of the Mn-S-state complex with polar amine vs other type compounds is discussed.

Introduction

Photosynthetic oxygen evolution proceeds via four successive one-electron oxidation steps in which two water molecules are oxidized to molecular oxygen [1,2]. These reactions which are well characterized kinetically are catalyzed by a polynuclear Mn-complex [3,4]. The number of Mn atoms within the complex, their valency state changes accompanying the linear transition of states S_0 to S_4 plus oxygen, and the precise identity of the protein/lipid components providing the proper configuration of the Mn atoms require the efficient oxidation of water remai solved. The old [4,5] and some of [6,7] literature argues for a

---

Abbreviations: PS, Photosystem: Chl, chlorophyll: Mn-S-state enzyme, the Mn-containing water-oxidizing complex of photosynthesis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid: Mes, 4-morpholineethanesulfonic acid: DCIP and $DCIPH_2$, the oxidized and reduced form of 2,6-dichlorophenolindophenol, respectively; TMPD, *N,N'*-tetramethyl-*p*-phenylenediamine; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TMF-2, Triton-prepared PS II membrane fragments.

some lines of evidence exist [8–10] indicating that at least a fraction of the Mn atoms within the complex at ground state is at a valency of more than 2^+.

A remarkable characteristic of the Mn-water oxidizing complex in chloroplasts and algae is its apparent low reactivity to many redox reagents. Considerable difficulty has been encountered in attempts to modify chemically the normal S_1/S_0 abundance in dark adapted material [11–14]. Only a few simple molecules such as H_2O_2 [14], NH_2OH [12,13] and NH_2NH_2 [14], or specific derivatives of these compounds [15], readily react with the S_1-state. Additionally, in chloroplasts only NH_3 and certain primary amines [16] react with the S_2- or S_3-states thereby inhibiting their advancement to higher S states and the release of molecular oxygen.

A plausible hypothesis to explain the high apparent reactivity of the Mn-water-oxidizing enzyme with water, but low reactivity with only certain simple molecules has been offered by Radmer and Ollinger [15]. According to their arguments, the Mn-S-state enzyme is buried within a crypt and topographic barriers prohibit free access of most chemicals to the enzyme. This deduction was based in part on differing capacities of variously substituted derivatives of NH_2OH and hydrazine to delay $Y_{O_2}^{max}$, the maximum yield of O_2 by a saturating light flash, in a sequence of flashes from the third to the fifth flash in dark-equilibrated chloroplasts [15].

The specificity observed among the hydroxylamine and hydrazine derivatives for delaying $Y_{O_2}^{max}$ by two flashes is not dissimilar, where intercomparisons can be made, to the specificity of such compounds for the inactivation of the Mn-S-state enzyme with a perturbation of the ligands binding the tetra-Mn polynuclear cluster [5]. Apparently, this inactivation process which results in solubilization of the ligated Mn [4,5] proceeds via the S_0-state [17], but is readily reversed in vivo by the process of photoactivation of the enzyme [16] in which Mn^{2+} is reincorporated into the polynuclear Mn-complex.

Analyses of specific mutants of *Scenedesmus* *Chlamydomonas* [19], and extraction/ alyses of O_2-evolving Triton-pre- fragments (TMF-2) [19–22], cholate extracted thylakoids [23,24], and PS 2 enriched inside-out thylakoids [25] have implicated the 17, 23 and 33 kDa 'extrinsic' polypeptides and the 34 kDa 'intrinsic' polypeptide in the binding and/or stabilization of the polynuclear Mn complex.

Here we report studies obtained using TMF-2 preparations on the mechanism of NH_2OH inactivation of O_2 evolution and the contributions of the 17, 23 and 33 kDa polypeptides to the postulated cryptic, microcompartment of the water oxidizing Mn-S-state enzyme. A preliminary report of some of these studies has been published elsewhere [19].

Materials and Methods

Chloroplasts and PS II-membrane preparations

Chloroplasts were prepared from 7–9 days old wheat seedlings grown in vermiculite in the greenhouse. The leaves were cut with scissors into approx. 3 mm segments then 70 g were blended 20 s in 450 ml buffer A (0.35 M sucrose/50 mM Hepes-NaOH/2 mM $MgCl_2$, pH 7.5) containing 0.1% bovine serum albumin and 0.2% sodium ascorbate. The homogenate was filtered through four layers of cheesecloth then centrifuged (3000 $\times g$ for 4.5 min). Following resuspension of the pellets in buffer A and centrifugation (1200 $\times g$/20 s), the chloroplasts were pelleted (3000 $\times g$/4.5 min). TMF-2 were then prepared by modifications of procedures described in Refs. 21 and 26. NaCl-TMF-2 completely depleted of the 17 and 23 kDa polypeptides were prepared by NaCl extraction during treatment of chloroplasts with Triton X-100 (details to be published elsewhere). The pelleted membranes were washed (approx. 1 mg Chl/ml) repeatedly with buffer B (15 mM NaCl/5 mM $MgCl_2$/20 mM Mes-NaOH, pH 6.2) containing 0.1% sodium ascorbate until the supernatants of the washes were devoid of Chl. The membranes were then resuspended and homogenized in buffer B containing 0.4 M sucrose before freezing (more than 2 mg Chl/ml) at −80°C. Unless otherwise noted the membranes were washed (approx. 1 mg Chl/ml) once and resuspended in buffer A before use. This wash diminished V_{O_2}, the rate of O_2 evolution, and Mn abundances of the membranes less than 15%, but removed some soluble protein.

$CaCl_2$-TMF-2 were prepared essentially as described by Ono and Inoue [22] from either freshly prepared or frozen TMF-2 and resuspended in 50 mM Mes-NaOH buffer (pH 6.5) containing 50 mM $CaCl_2$. Unless otherwise noted, the $CaCl_2$-TMF-2 were prepared just prior to use.

Rate measurements of V_{O_2} and DCIP photoreduction

V_{O_2} measurements were made polarographically essentially as described previously (4). Light from a 500 W projection lamp was filtered through 5 cm of 2% $CuSO_4$, two Schott 116 and an OG-3 filter, then focused with suitable condensing lenses onto the vessel of 1.0 ml capacity. The light intensity was just saturating for assays containing 0.4 M sucrose/50 mM Mes-NaOH/15 mM NaCl (pH 6.2), 300 μM phenyl-*p*-benzoquinone, 1 mM FeCN and TMF-2 equivalent to 5 μg Chl per ml.

DCIP photoreduction was measured at 580 nm with an Aminco-DW-2 spectrophotometer. Side illumination was provided with a Dolan-Jenner Series 180 high intensity illuminator. The light was focused onto the cuvette by fiber-optics and filtered through a Schott 116 and a Corning 2-59 filter. A Corning 4-95 filter masked the photomultiplier tube. The reaction mixture contained 50 mM Mes-NaOH (pH 6.5)/40 μM DCIP/CaCl-TMF-2 equivalent to 10 μg Chl per ml and various concentrations of artificial PS II e^- donors as noted. Absolute rates were determined using 13.8 $mM^{-1} \cdot cm^{-1}$ at pH 6.5 as the millimolar extinction coefficient for DCIP.

Measurements of effects of NH_2OH and related compounds on V_{O_2} and solubilization of protein and functional Mn

TMF-2 (1 mg Chl/ml) were incubated for 30 min at 4°C in the absence or presence of NH_2OH (or related compounds), as designated in individual figures, then centrifuged (27000 × *g*/10 min). The pellets were washed twice (buffer A, approx. 1 mg Chl/ml) and the supernatants combined. Amounts of solubilized protein in the supernatants were determined either directly or following dialysis (6000–8000 MW cut-off tubing) vs, 5 mM Mes-NaOH (pH 6.5), and subsequent lyophilization. More total protein (approx. 2–2.5-fold) was detected in the direct determinations despite all attempts to minimize losses during dialysis and lyophilizations.

The TMF-2 pellets were resuspended in buffer B containing 0.4 M sucrose for assays of V_{O_2} and Mn abundance (indirect determination procedure).

In experiments where we measured only the effects of NH_2OH and related derivatives on V_{O_2} (direct determination procedure), TMF-2 and NaCl-TMF-2 were washed and resuspended in 0.4 M sucrose/15 mM NaCl and 50 mM Mes-NaOH (pH 6.5) or Hepes-NaOH (pH 7.5), depending on the particular experiment. In the pH dependence experiments, the washed membranes were resuspended (2–3 mg Chl/ml) similarly, except the buffer contained 5mM Mes-NaOH (pH 6.5). Hydroxylamine or related derivatives, freshly prepared in 0.4 M sucrose/15 mM NaCl and 50 mM buffer at the appropriate pH value was then added to the membranes to yield 500 μg Chl/ml and the designated concentration of NH_2OH or related derivative. After incubation in darkness at 4°C for the designated time, the remaining V_{O_2} was determined by injecting an aliquot (15 μl) of the suspension into a polarographic vessel of 1.0 ml capacity and immediately assayed. The buffers used in incubations at various pH values were Mes, Hepes and Tricine for pH values less than 7, in between 7 and 8, and more than 8, respectively.

Reconstitution of NaCl-TMF-2 with 17 and 23 kDa polypeptides

A 17 and 23 kDa protein fraction [25] was obtained by extraction of TMF-2 (0.5 mg Chl/ml) with 20 mM Mes-NaOH/5 mM $MgCl_2$/2 M NaCl for 1 h at 4°C. Following centrifugation (30000 × *g*/20 min), the supernatant was concentrated and desalted (25 mM Mes-NOH, pH 6.1) using an Amicon PM-10 membrane then recentrifuged. the proteins were dialyzed using 5 mM Mes-NaOH, pH 6.4, centrifuged, concentrated by lyophilization, and redialyzed using 5 mM Mes-NaOH (pH 6.4), before use or freezing (−80°C). SDS-polyacrylamide gel electrophoresis analyses of this fraction showed only minor contamination by polypeptides of 33 kDa and of up to 10 kDa.

Preliminary experiments showed that effective reconstitution of V_{O_2} of NaCl-TMF-2 by the 17 and 23 kDa proteins required low-ionic conditions. Accordingly, NaCl-TMF-2 were washed and resuspended in 0.4 M sucrose/15 mM NaCl, and only 5 mM buffer before addition of the 17 and 23 kDa protein fraction (1.34 μg protein/μg Chl). Maximum and equivalent effect on V_{O_2} from these proteins was obtained by 5 min incubation at 4°C at either pH 6.5 (Mes-NaOH), pH 7.0 or pH 7.5 (Hepes-NaOH). Thus in the experiments of Fig. 7, the membranes reconstituted at these pH values were used directly after the 5 min incubation to avoid slow solubilization occurring at pH 7.5 or more, of the 17 and 23 kDa proteins from the reconstituted membranes. The results obtained in this way were entirely similar to those obtained with NaCl-TMF-2 reconstituted at pH 6.5, but centrifuged and resuspended in buffer of the appropriate pH before the experiments of Fig. 7.

Mn determinations

All glassware was washed in a 1 : 1 (v/v) mixture of 1 M HNO_3 and 1 M HCl and rinsed with glass-distilled H_2O. Samples (standards and TMF-2 equivalent to 100–500 μg Chl, depending on Mn abundance) were digested in test tubes covered with watch glasses with 0.3 ml of a mixture of concentrated HNO_3 and 70% $HClO_4$ (9 : 1, v/v) for 2 h at 98°C, 3 h at 198°C, then 2 h at 220°C. Watch glasses were removed after 2 h at 98°C. After cooling, 2.5 ml of 4 mM HNO_3 containing 10 mm HCl was added, the tube tops parafilmed, then heated 12 h at 98°C. Sample and water rinses were combined, the volume adjusted to 5 ml with water then centrifuged before analyses by flameless atomic absorption. Absorbance (279.46 nm) versus Mn concentration was linear up to at least 50 ng Mn/ml (absorbancy value of approx. 0.7).

Other methods

Protein content of TMF-2 preparations was determined by the method of Lowry et al. [27] with deoxycholate (0.1%, w/v). Soluble proteins were determined using the Bradford reagent [28]. SDS-electrophoresis analyses were done essentially as described by Chua [29] using 5% and 12% polyacrylamide gels for stacking and running, respectively.

Crosslinking of TMF-2 and NaCl-TMF-2 was done with dithiobis(succinimidylpropionate), of which solutions (100 mM) were freshly prepared in dimethylsulfoxide, then 20 μl additions were made repetitively to the membranes (500 μg Chl/ml) with mixing until 2.5 mM dithiobis(succinimylpropionate) was obtained. After 45 min incubation in buffer A at 4°C, the membranes were recovered by centrifugation, washed twice with buffer B containing 0.4 M sucrose before final resuspension in this same buffer.

Results

Several chemically unrelated compounds are known to affect rather specific inactivation of the water-oxidizing S-state enzyme of chloroplasts and release of its Mn [16]. Using PS II-enriched inside-out thylakoids [25] and/or Triton-prepared O_2 evolving PS II membranes [21,26], a number of laboratories have shown such compounds additionally solubilize, with differing extents and specificities, the PS II 17, 23 and 33 kDa polypeptides [19–25]. In few cases have publications appeared in which chemically induced V_{O_2} inactivation, Mn and protein solubilization have been examined in detail. Hydroxylamine is the most effective of all the chemical inactivators of the Mn-S-state enzyme as evidenced by low concentration requirements but modifiable by N- and O-substitutions of the molecule [5,15]. Yet the mechanism of action of NH_2OH is poorly defined [15,16].

Mn and protein solubilization from TMF-2 by NH_2OH: effects on V_{O_2}

Fig. 1 shows the effect of incubation (30 min in buffer A) of TMF-2 with increasing NH_2OH concentrations before recovery of membranes and assays of V_{O_2} (main figure) and remaining Mn abundance (inset). Preliminary experiments (see Fig. 6) showed that this pH was optimal for maximum effect of NH_2OH on TMF-2, as previously reported for chloroplasts [5], and yielded only approx. 5% loss of V_{O_3} in controls. The data shows that only 100 μM NH_2OH (NH_2OH/Chl of 0.1) sufficed to yield 50% inactivation of the S-state enzyme centers. The inset of Fig. 1 records V_{O_2} vs. Mn abundance of TMF-2 subjected to the NH_2OH extractions shown in the main figure. These data show a linear relationship between V_{O_2} and Mn abundance between the limits of 0.3–7.5 and 0.7–9.2 Mn/400 Chl in these two experiments and

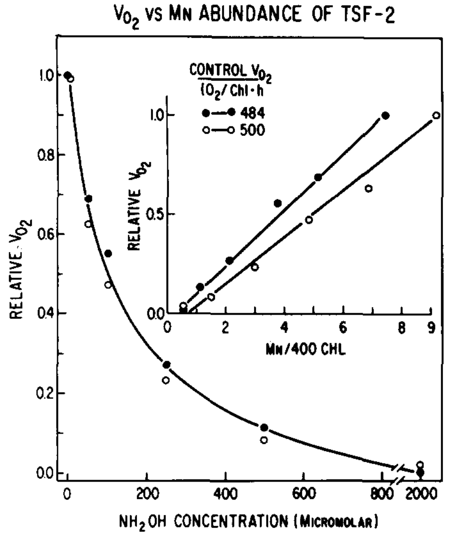

Fig. 1. NH_2OH concentration dependency for inactivation of V_{O_2} with TMF-2 (TSF-2) membranes. Inset: The relationship between V_{O_2} and bound Mn of TMF-2. Open and closed circles represent TMF-2 with 500 and 484 μmol O_2/mg Chl per h, respectively. TMF-2 membranes (1 mg Chl/ml) were incubated at 4°C for 30 min in 4 ml buffer A containing 20 μM ionophore A23187, 1 mM EDTA and the NH_2OH concentrations noted. The suspension was then diluted 2-fold with buffer A, before pelleting of the membranes. The membranes were washed (1 mg Chl/ml) with buffer A, containing 20 μM ionophore A23187 and 1 mM EDTA before resuspension in buffer B, containing 0.4 M sucrose. Omission of ionophore A23187 and EDTA had no effect on V_{O_2}, but resulted in 12% greater Mn abundances in the extracted membranes.

are similar to previous data obtained with chloroplasts [4,5] except that the TMF-2 Mn abundance correlating with V_{O_2} is approx. 2-fold greater. This conclusion is reinforced from measurements of functional Mn of the chloroplasts from which TMF-2 (open circles) was prepared. These chloroplasts contained 5.1 Mn/400 Chl which was diminished to 0.5 Mn/400 Chl with loss of V_{O_2} by extraction with 2.5 mM NH_2OH in the presence of 2.0 mM EDTA and 20 μM ionophore A23187. This approx. 2-fold enrichment of Mn in TMF-2 relative to chloroplasts has been reported earlier [19], and again more recently by others [30]. Assuming PS II units of 400 and 200 Chl for the chloroplasts and TMF-2 preparations, respectively, and perfect Mn-S-state enzyme complexes, the data again suggest a tetra-Mn polynuclear S-state enzyme [4,7].

Fig. 2 shows that the low NH_2OH concentrations sufficient for inactivation of the S-state enzyme with solubilization of its Mn also yield significant solubilization of protein from the TMF-2 preparations. In the experiment shown in Fig. 2 where we directly analyzed soluble protein in supernatants of the extractions, 125 and 2000 μM NH_2OH solubilized 260 and 440 μg protein, respectively, over the amount in controls (100 μg protein from 4 mg Chl, equivalent to approx. 20 mg protein). In similar incubations, 0.8 M Tris (pH 8.0) solubilized 1000 μg protein over the amount in controls.

These amounts of protein solubilized by NH_2OH are approx. 2-fold greater than reported previously [19]. As shown in the inset of Fig. 2, only approx. 100 μM NH_2OH was sufficient for half-maximal solubilization of functional Mn and protein and the inactivation of V_{O_2}. The very similar and low NH_2OH concentration dependency to obtain these effects coupled with the low total amount of protein solubilized (approx. 2%) suggest highly specific and interrelated effects of NH_2OH on the Mn-S-state enzyme. On the other hand, Yocum et al. [31] have indicated inactivation

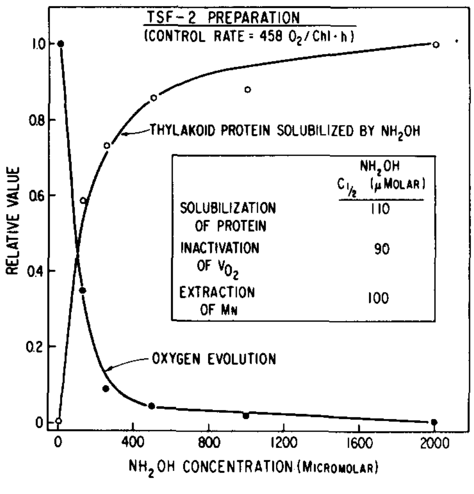

Fig. 2. Comparison of NH_2OH concentration dependency for inactivation of V_{O_2} vs. solubilization of protein from TMF-2 (TSF-2) membranes. After incubation (see legend of Fig. 1), the membranes were recovered by centrifugation without prior dilution. Following resuspension (1 mg Chl/ml) with buffer A, and a 10 min incubation, the membranes were recovered. Protein determinations were made directly on the combined supernatants. See text for other details.

of V_{O_2} ($t_{1/2} > 3$ h) by 100 μM NH_2OH (pH 6.0) with neither solubilization of Mn nor PS II extrinsic polypeptides.

We observed the maximum amount of protein solubilized by 2.0 mM NH_2OH extraction (in buffer A) varied between 3–10-fold over the amount solubilized by buffer A in different preparations. Generally, our data show: (1) TMF-2 with high V_{O_2} activity (500–700 O_2/Chl per h) and high abundances of the 17, 23 and 33 kDa polypeptides yield more protein solubilized specifically by NH_2OH extraction than TMF-2 having low V_{O_2} activity: an (2) the amount of protein solubilized by 0.8 M Tris extraction is approx. 2 fold greater than by either 2 mM NH_2OH or 1 M NaCl in buffer A.

SDS electrophoresis of TMF-2 preparations and proteins solubilized by NH_2OH and Tris

The polypeptides solubilized by 1 mM NH_2OH during 30 min extraction of TMF-2 (lane A) and TMF-2 previously subjected to 1 M NaCl extraction to deplete the 17 and 23 kDa polypeptides [19] (lane B) are shown in lanes e and f, respectively, of Fig. 3. Shown also in Fig. 3 are the SDS electrophoresis profiles of TMF-2 subjected to one (lane C) or two (lane D) successive 1 M $CaCl_2$ extractions [22] (see later). Lane G shows the 17, 23 and 33 kDa proteins solubilized by 30 min extraction of TMF-2 with 0.8 M Tris (pH 8.0) [19–21]. The NH_2OH extractions of TMF-2 and NaCl extracted TMF-2 were done at pH values (pH 7.5 and 6.5, respectively) optimal for inactivation of V_{O_2} for two type TMF-2 preparations (see Fig. 6). Comparison of lanes E vs. G shows that extraction of TMF-2 with either 1 mM NH_2OH or 0.8 M Tris resulted in a solubilization of the 17, 23 and 33 kDa polypeptides. Since equivalent amounts (25 μg) of proteins were loaded in lanes E and G, we conclude that NH_2OH solubilized principally the 17 and 23 kDa and to a lesser extent the 33 kDa polypeptide. As shown in lane F (4.5 μg protein load), NH_2OH extraction of TMF-2 pre-extracted with 1 M NaCl solubilized principally the 33 kDa polypeptide.

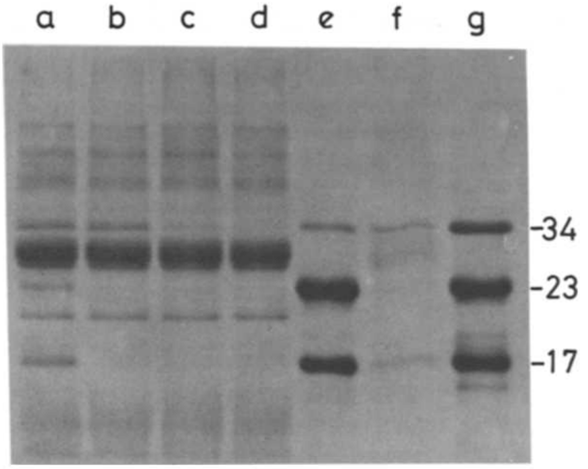

Fig. 3. SDS-polyacrylamide gel electrophoresis analyses of TMF-2 preparations and proteins solubilized by extraction with NH_2OH and Tris. Lane A: TIMF-2: lane B: TMF-2 extracted with 1 M NaCl: lanes C and D: TMF-2 extracted once and twice, respectively, with 1 M $CaCl_2$: lane E: protein (25 μg protein load) solubilized by 1 mM NH_2OH extraction (30 min) of TMF-2: lane F: protein (4.5 μg protein load) solubilized by 1 mM NH_2OH extraction of TMF-2 preextracted with 1 M NaCl; lane G: protein (25 μg protein load) solubilized by 0.8 M Tris (pH 8.0), extraction of TMF-2. THe load in lanes A–D was 10 μg Chl. The stacking and running gel contained 5 and 12% polyacrylamide, respectively.

Though NH_2OH does affect solubilization of these PS II extrinsic proteins, the protein solubilization is secondary to Mn solubilization in the inactivation of the S-state enzyme: (1) one NH_2OH extraction suffices to solubilize Mn, but even repeated NH_2OH extractions do not totally solubilize all the 17, 23 and 33 kD peptides; (2) Tris extraction of TMF-2 previously extracted with 2.5 mM NH_2OH gave virtually complete solubilization of the remaining 17, 23 and 33 kDa polypeptides with the 33 kDa species predominant in the supernatant. In contrast, neither subsequent extraction with 500 mM NaCl nor 2.5 mM NH_2OH solubilized appreciably more protein: and (3) TMF-2 preparations crosslinked with dithiobis-(succinimidylpropionate) (2.5 mM for 45 min) still retained 57% V_{O_2}, but were inactivated with loss of Mn by NH_2OH (or Tris) extraction without appreciable solubilization of protein(s).

In the following sections, we asked if the 17, 23 and 33 kDa polypeptides affected the reactivity of the S-state enzyme to chemical analogs of water molecules – i.e., NH_2OH, NH_2NH_2 and their various substituted derivatives [15]. Meaningful analyses required kinetic characterization of the inactivation process induced by such chemicals.

Mechanism of NH_2OH action: kinetics and tests for cooperativity

Fig. 4A shows the effect of time of extraction with NH_2OH concentrations at pH 7.5 on V_{O_2} of TMF-2. Fig. 4B shows the effect of pH on the inactivation of V_{O_2} of TMF-2 by 500 μM NH_2OH or 5 mM CH_3NHOH. The data show: (1) at less than 2 mM NH_2OH, the inactivation of V_{O_2} obeys simple apparent first-order kinetics with the rate constant dependent on NH_2OH concentration (Fig. 4A): (2) the inactivation process is highly dependent on pH for either NH_2OH or CH_3NHOH and at optimum pH (approx. pH 7.5) about an 8-fold higher concentration of CH_3NHOH than NH_2OH is required to give similar rates of inactivation (Fig. 4B): and (3) with NH_2OH concentrations of more than 2 mM, biphasic inhibition of V_{O_2} occurs as reported previously with chloroplasts [5] and *Chlorella* [32]. The V_{O_2} inactivation rate constant dependency on pH for NH_2OH and CH_3NHOH is entirely similar to that reported with chloroplasts [5]. With both chloroplasts and the TMF-2, the inactivation of V_{O_2} is dependent on the unprotonated species of NH_2OH ($pK_a = 5.97$) and CH_3NHOH ($pK_a = 5.96$) [33]. With chloroplasts such dependency was postulated to reflect chloroplast thylakoid permeability to only the unprotonated species of NH_2OH. Since TMF-2 apparently have an inside-out orientation [34], the results shown in Fig. 4B must directly reflect specific requirements in the reaction between the Mn-S-state enzyme and NH_2OH or CH_3NHOH.

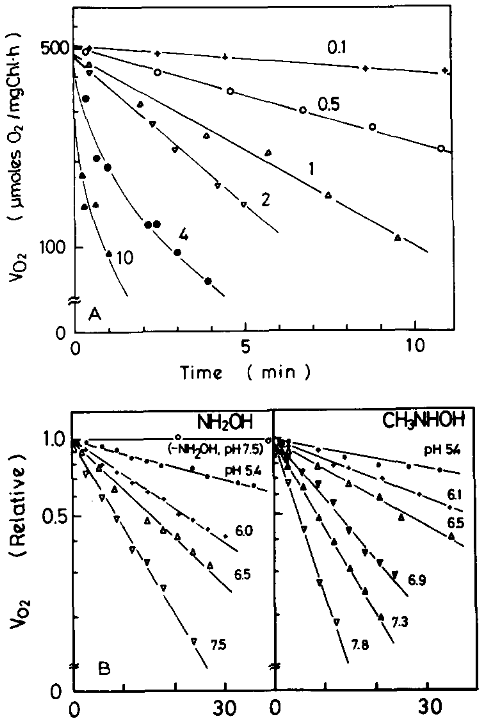

Fig. 4. Dependency of rate of inactivation of V_{O_2} on NH_2OH concentration (mM) at pH 7.5 (A) and the pH dependency of inactivation by 500 μM NH_2OH and 5 mM CH_3NHOH (B). The direct determination procedure (Materials and Methods) was used with TMF-2 membranes. Assays of V_{O_2} were made at pH 6.2 (Materials and Methods).

The fast phase in inhibition of V_{O_2} observed with more than 2 mM NH_2OH is possibly attributable to a direct, reversible [35] inhibition of electron transfer between PS II reaction center chlorophyll, P-680, and primary donor to P-680, Z [32] by a reaction presumably independent of the slower reactions of NH_2OH with the S-state enzyme. However, in experiments similar to those of Fig. 4A in which TMF-2 were incubated for 15–60 s with 10 mM NH_2OH, diluted 100-fold with buffer A, then recovered by centrifugation, no evidence could be obtained for any reversible inhibition action of NH_2OH. It is likely the fast phase of inhibition of V_{O_2} includes irreversible inactivation of the Mn-S-state enzyme in addition to the reversible inhibition at the P-680-Z locus.

At micromolar concentrations, NH_2OH competes with water as the electron donor to the Mn-S-state enzyme [12–15] as evidenced by a Y_3–Y_5 shift, but then subsequent return to normal oscillation of period four in Y_{O_2} measurements. This process apparently proceeds either by reduction of S_1 to S_0 with subsequent binding of one NH_2OH molecule [15] or the binding of two NH_2OH molecules to S_1, possibly involving a cooperative type mechanism [13]. Data in the literature indicate that the separation of effects of NH_2OH on the Y_3–Y_5 shift from the inactivation of the S-state complex may be kinetically possible, but that both processes may be similar mechanistically. Though light inhibits NH_2OH-induced inactivation of the S-state complex by NH_2OH, this observation [17] does not distinguish between the two alternative hypotheses for the reaction(s) of

NH_2OH with the S-state enzyme or discriminate between a non-cooperative vs. a cooperative type mechanism.

Fig. 5 shows the dependence of the inactivation rate constant on the NH_2OH concentration with TMF-2 and NaCl-TMF-2 at pH 6.5 and 7.5, and over a range of NH_2OH concentrations (50 μM–10 mM).

Over the range of NH_2OH concentrations studied, the inactivation rate constants at pH 7.5 were greater than those at pH 6.5 with TMF-2: however, with NaCl-TMF-2 this pH dependency was reversed (see Fig. 6). The rate constant dependency on NH_2OH concentration showed no sigmoidicity over the range of 50–1200 μM ($[NH_2OH]/[P\text{-}680]$ of 25–600) with TMF-2 or NaCl-TMF-2 at either pH 7.5 or pH 6.5. Above approx. 2 mM NH_2OH where biphasic inactivation kinetics were observed, the rate constants appeared to increase sigmoidally (Fig. 5 inset): however, for technical reasons, we could not determine precisely the NH_2OH concentration just yielding the maximum rate of inactivation. Note the high value of the rate constant (approx. 2.8 min^{-1}, corresponding to $t_{1/2}$ of aprox. 15 s) observed with TMF-2 and 10 mM NH_2OH.

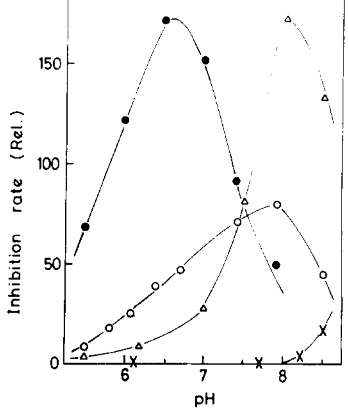

Fig. 6. Relative rate constants of inactivation of V_{O_2} versus pH with TMF-2 (△, ○) and NaCl-TMF-2 (●). The data of open and closed circles were obtained using 500 μM NH_2OH. △, data were obtained using 500 μM NH_2NH_2. The symbols X represent inactivation occurring in the absence of NH_2OH or NH_2NH_2 in TMF-2. The direct determination procedure (Materials and Methods) was employed using 0.5 mg Chl/ml in the incubations.

At less than 1 mM NH_2OH, no evidence was obtained for a cooperative mechanism in the inactivation of the Mn-S-state enzyme of either TMF-2 or NaCl-TMF-2. On the other hand, the sigmoidal behavior of the rate constants at more

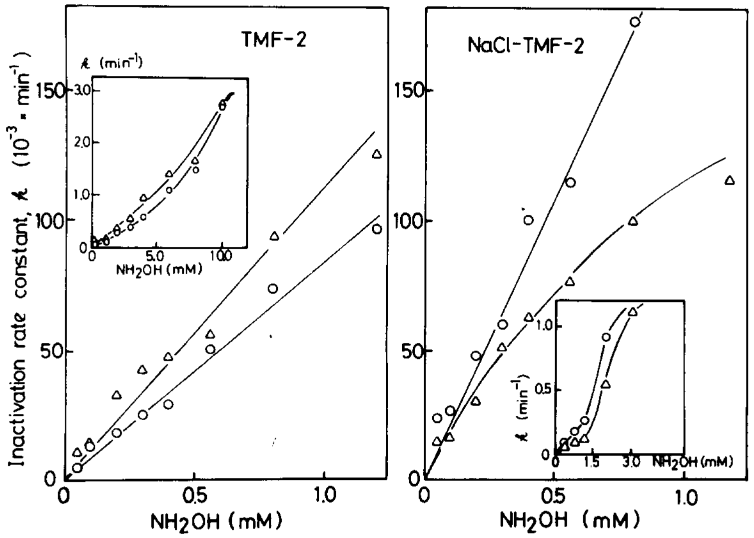

Fig. 5. Dependence of inactivation rate constant (k) on NH_2OH concentration in TMF-2 and NaCl-TMF-2 at pH 7.5 (△) and 6.5 (○). Inactivation rate constants (min^{-1}) were calculated from $\ln 2/t_{1/2}$. Insets represent dependence of k on NH_2OH at high concentrations (up to 10 mM).

than 2 mM NH_2OH may reflect an appearance of a cooperative inactivation of the Mn-S-state enzyme. If so, this inactivation appears different from the proposed cooperative binding of NH_2OH to the Mn-S-state enzyme [13] and unrelated to the inhibition of the P-680-Z locus by NH_2OH [35].

Effects of the 17 and 23 kDa polypeptides on rate constants and pH dependency of NH_2OH and NH_2NH_2 inactivation of V_{O_2}

The literature suggests that the 17 and 23 kDa extrinsic PS II polypeptides are closely associated with the S-state water oxidizing enzyme but are not absolutely essential for V_{O_2} (Ref. 19 and 37: see, however, Refs. 25, 38, 39). In the following sections we asked if the extrinsic PS II polypeptides (17, 23 and 33 kDa) contributed to the topography surrounding the cryptic S-state water oxidizing enzyme and thereby affected the reactivity of this enzyme with certain highly polar and weakly basic chemical reagents postulated [15] not to react with this enzyme because of steric restraints.

Fig. 6 shows pH dependency of the first-order rate constant of inactivation of V_{O_2} caused by 500 μM NH_2OH with TMF-2 (○) and NaCl-TMF-2 (●). The complete extraction of the 17 and 23 kDa polypeptides yields only approx. 50% decrease of O_2 evolution in saturating light [19], in weak light or in Y_{O_2} measurements (unpublished data). Also shown in Fig. 6 is the pH dependency of the first-order inactivation rate constant caused by 5 mM NH_2NH_2 ($pK_a = 8.07$) [40] with TMF-2 (△).

We note that the rate constants of inactivation of V_{O_2} in TMF-2 by NH_2OH and NH_2NH_2 are maximum at pH 7.8 and 8.0, respectively, then decline with increasing pH values. The decrease in the inactivation rate constants at the more alkaline pH (more than 8.0) may reflect an instability of NH_2OH and NH_2NH_2 at alkaline conditions. The data of Fig. 6 therefore indicate that only the unprotonated and semi-protonated species of NH_2OH and NH_2NH_2, respectively, are effective in the inactivation process of TMF-2.

Furthermore, the data of Fig. 6 show that the rate constant of inactivation of V_{O_2} by NH_2OH and its dependency on pH is strongly affected by the polypeptide composition of TMF-2: the depletion of the 17 and 23 kDa polypeptides results in a shift in the optimum pH for inactivation of V_{O_2} (pH 6.5 vs. 7.8 for NaCl-TMF-2 and TMF-2, respectively) and an increase in the maximum inactivation rate constant by an approx. 2-fold relative to that obtained with TMF-2.

The observed diffrent behaviors of the two preparations to NH_2OH are not a consequence of differing stabilities of V_{O_2} in the absence of NH_2OH at the conditions of the experiment. Additionally, the observed differences cannot be explained on the basis of differing local pH domains [41] in PS II of the two type preparations, since gramacidin D (up to 100 μg per 450 μg Chl) affected neither the control rates of V_{O_2}, the pH dependencies of inactivation nor the rate constants of inactivation. We therefore argue from the data of Fig. 6 that the 17 and 23 kDa polypeptides directly affect the reaction(s) of NH_2OH with the S-state enzyme.

Previous data [39,42,43] indicate that rebinding of the 17 and 23 kDa polypeptides to 1 M NaCl extracted PS II membranes or addition of high $CaCl_2$ concentrations to such extracted membranes results in at least partial restoration of V_{O_2} lost by the extraction.

In the experiments summarized in Fig. 7, we asked if the reconstitution of NaCl-TMF-2 membranes with the 17 and 23 kDa polypeptides or the addition of high $CaCl_2$ concentrations 'reversed'

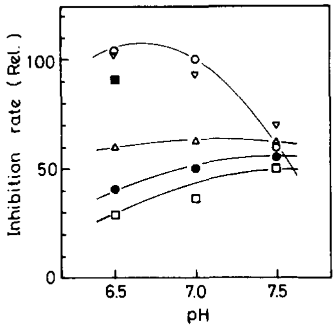

Fig. 7. Effects of Ca^{2+} and reconstitution of NaCl-TMF-2 membranes with 17 and 23 kDa proteins on the relative rate constants of inactivation of V_{O_2} by NH_2OH with NaCl-TMF-2. Data of open and closed circles were obtained with NaCl- and TMF-2, respectively: open squares with 17 and 23 kDa reconstituted NaCl-TMF-2 (1.34 mg protein per mg Chl): triangles and inverted triangles with NaCl-TMF-2 in the presence of 150 mM $CaCl_2$ and 150 mM $MgCl_2$, respectively: and closed square with bovine serum albumin (1.34 mg protein per mg Chl). See Materials and Methods for other details.

the NH_2OH reaction characteristics of NaCl-TMF-2 to those of TMF-2 having normal abundance of these polypeptides. The reconstitution procedures employed (Materials and Methods) routinely yielded a 1.5–1.6-fold enhancement of V_{O_2} of NaCl-TMF-2 corresponding to 75–85% of V_{O_2} of TMF-2. On the other hand, the $CaCl_2$ (150 mM per 500 μg Chl/ml) enhancement of V_{O_2} of NaCl-TMF-2 generally proved less (30–40% enhancement of V_{O_2}) in different experiments.

The data of Fig. 7 show that these effects of reconstitution or $CaCl_2$ additions on V_{O_2} of NaCl-TMF-2 are qualitatively reflected in changes in the reaction(s) of NH_2OH with the S-state enzyme. We note: (1) the optimum pH for the maximum inactivation rate constant of the S-state enzyme in NaCl-TMF-2 (○) is shifted from pH 6.5 to 7.8 following reconstitution with the 17 and 23 kDa polypeptides (□) and the pH profile of the inactivation rate constant with reconstituted membranes is entirely similar to TMF-2 (●); (2) reconstitution with the 17 and 23 kDa polypeptides decreases the rate constant measured at pH 6.5 by approx. 3.5-fold and the effect given by these PS II proteins could not be obtained with bovine serum albumin (■) having no effect on V_{O_2} of the NaCl-TMF-2: and (3) a high $CaCl_2$ concentration (△), which was less effective than the 17 and 23 kDa polypeptides in enhancing V_{O_2} of NaCl-TMF-2, yielded intermediate effects on the pH profile and value of the inactivation rate constant of the S-state enzyme: moreover, $MgCl_2$, which did not affect V_{O_2} of NaCl-TMF-2, also did not change the reactivity of NH_2OH with the S-state enzyme in NaCl-TMF-2.

Clearly, the results shown in Figs. 6 and 7 show that the 17 and 23 kDa polypeptides specifically affect and modify the reaction(s) between the S-state enzyme and NH_2OH. Such effects of these polypeptides appear to be closely related to their effects on V_{O_2}.

Contributions of the PS II extrinsic polypeptides to the Mn-S-state enzyme topography and its reactivity to water analogs

Preceding subsections gave evidence that the interaction of NH_2OH with the Mn-S-state enzyme resulted in total solubilization of functional Mn, at least partial solubilizations of the 17, 23 and 33 kDa polypeptides, and that the 17 and 23 kDa polypeptides were determinants of the reactivity of NH_2OH with this water oxidizing enzyme. We previously gave evidence [19] indicating a close correlation between the effectivity of various substituted NH_2OH/NH_2NH_2 derivatives for inactivation of V_{O_2} vs. Mn and protein solubilization from TMF-2. Accordingly, in the experiments of Fig. 8 we asked if the 17, 23 and 33 kDa polypeptides contributed structurally to the crypt postulated [15,19] to limit sterically the reaction of bulky derivatives of the highly polar, weakly basic NH_2OH/NH_2NH_2 compounds with the buried Mn-S-state enzyme.

Neither the extraction of the 17 and 23 nor the extraction of the 17, 23 and 33 kDa polypeptides greatly diminished the Mn abundance (approx. 7.2 Mn/400 Chl) of the parent membranes but inhibited V_{O_2} by approx. 50 and 80%, respectively. The somewhat diminished Mn abundance in controls for NaCl- and $CaCl_2$-TMF-2 are attributed to the several prewashings and the subsequent washings following the incubations with the compounds noted. In the experiments of Fig. 8A, we used various concentrations of the different polar amine-type compounds ranging from 100 μM to 10 mM depending on the effectivity of the various compounds for inactivation of V_{O_2} with TMF-2. The same concentration of each compound then was generally used in the extractions of the NaCl- and $CaCl_2$-TMF-2. All extractions for all three types of TMF-2 were done at pH 7.5, a condition not necessarily optimal for each of the compounds or for each type of TMF-2 preparations: nevertheless, meaningful comparisons can be made from the data of Fig. 8A.

A hierarchy of effectivity for inactivation of V_{O_2} and Mn solubilization is observed among these compounds with TMF-2: $NH_2OH > CH_3NHOH > NH_2NH_2$, $NH_2OSO_3 > NH_2OCH_3 \gg NH_4Cl$, CH_3NHOCH_3: and essentially the same hierarchy is observed also with NaCl- and $CaCl_2$-TMF-2, respectively. Additionally, in all types of TMF-2 preparations V_{O_2} appears to be linearly related to their Mn abundance irrespective of the V_{O_2} value before extraction with any of the amine compounds.

As shown in Fig. 8B, the same hierarchy is also observed in the relative effectivity of these com-

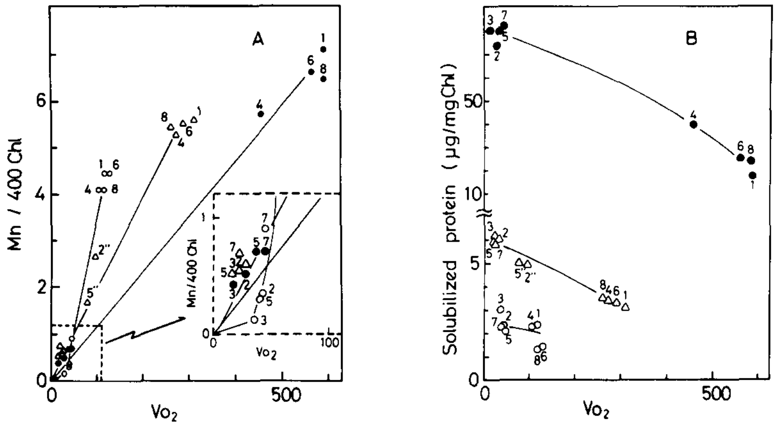

Fig. 8. Analysis of the effectivity of NH_2OH, NH_2OH derivatives, NH_2NH_2 and NH_4Cl for the inactivation of V_{O_2} and solubilization of Mn (A) and proteins (B) with TMF-2 (closed circles), NaCl-TMF-2 (△) and $CaCl_2$-TMF-2 (○). The numbers within the figure identify: (1), controls; (2) and (2″), 1 and 0.1 mM NH_2OH, respectively; (3), 5 mM CH_3NHOH; (4), 10 mM NH_2OCH_3; (5) and (5″), 1.0 and 0.1 mM NH_2OSO_3, respectively; (6), 10 mM CH_3NHOCH_3; (7), 10 mM NH_2NH_2: and (8), 10 mM NH_4Cl. The indirect determination procedure (Materials and Methods) was employed using 1.0 mg Chl/ml during 30 min incubations.

pounds for solubilization of protein from the TMF-2. This conclusion is revealed most clearly with TMF-2 and to a lesser extent with NaCl-TMF-2. SDS electrophoresis analyses of solubilized proteins revealed no qualitative differences among the proteins solubilized from TMF-2 by the various compounds.

The hierarchy among the compounds used in the experiments of Fig. 8 is similar where comparisons can be made to the hierarchy observed with chloroplasts [5] except for NH_2OSO_3 for inactivation of V_{O_2} and Mn solubilization and the delay of Y_{O_2} from Y_3 to Y_5 in dark-adapted chloroplasts [15] – e.g., $NH_2OH > CH_3NHOH$ with virtually no effect from 10 mm CH_3NHOCH_3.

The observed differing effectivity among NH_2OH and its derivatives (Fig. 8) relate either to their differing chemical reactivities with the Mn-water-oxidizing enzyme or perhaps to topographic barriers limiting access of the bulky substituted derivatives to this enzyme [15]. Their similar effectivities for inactivation of V_{O_2} and solubilization of Mn with TMF-2 preparations containing or lacking either the 17 and 23, or the 17, 23 and 33 kDa polypeptides tended to suggest that these extrinsic polypeptides did not comprise a topographic barrier for the reaction of these polar amine-type compounds with the Mn-water-oxidizing enzyme.

The data of Table I compare NH_2OH, CH_3NHOH and CH_3NHOCH_3 as e^- donors to PS II vs. their capacity to inactivate V_{O_2} in TMF-2 and $CaCl_2$-TMF-2 preparations. First, the data show that the photoreduction of DCIP by these compounds is about equivalent at sufficiently high concentrations of these compounds. We attribute the observed low rates to a sluggish reaction between DCIP and the PS II acceptor side. PS II acceptors other than primary and secondary acceptor quinones of PS II, PQ_A and PQ_B, are essentially depleted in these preparations (data not shown). Second, the data show that though CH_3NHOH is equivalent to NH_2OH as an e^- donor to PS II, based on the concentration dependency for half-maximal rate, a 5–8-fold higher concentration of CH_3NHOH than NH_2OH is required for inactivation of V_{O_2} in both TMF-2 and $CaCl_2$-TMF-2. On the other hand, a 25–30-fold higher concentration of CH_3NHOCH_3 than NH_2OH or CH_3NHOH is required for half maximal PS II donor activity yet we find this compound ineffective for inactivation of V_{O_2} and Mn solubilization in TMF-2 and $CaCl_2$-TMF-2 even at 250 mM concentrations. If the chemical reactivity of such compounds to PS II traps is equivalent to their reactivity with the water-oxidizing enzyme, the data of Table I and Fig. 8 suggest (a) steric factors limit the reactivity of *N*-methyl and *N,O*-

TABLE I

COMPARISON OF COMPOUNDS AS PS II DONORS VS. INACTIVATORS OF V_{O_2} IN TMF-2 AND $CaCl_2$-TMF-2

Values for PS II donor were determined with $CaCl_2$-TMF-2 with corrections for any photoreduction from H_2O. Concentration for half-maximal inactivation of V_{O_2} was determined by incubation, centrifugation and subsequent washing of membranes before assay. The Mn abundances in TMF-2 and $CaCl_2$-TMF-2 were 7.95 and 7.08 Mn/200 Chl, respectively, and were not diminished by 250 mM CH_3NHOCH_3

	PS II donor		Inactivation of V_{O_2}: concentration for half-maximal inactivation	
	Maximum rate (equiv./Chl per h)	Concentration for half-maximal rate (mM)	TMF-2 (mM)	$CaCl_2$-TMF-2 (mM)
NH_2OH	62	0.4	0.1	0.1
CH_3NHOH	46	0.3	0.8	0.5
CH_3NHOCH_3	54	10	– none by 250 mM –	

dimethylhydroxylamine with this enzyme, and (b) the depletion of the 17, 23 and 33 kDa PS II polypeptides does not appear to modify the steric constraints.

Table II gives evidence, however, that the 17, 23 and 33 kDa polypeptides do affect the reactivity of the water-oxidizing enzyme with NH_2OH. Here we determined second order rate constants for the NH_2OH-induced inactivation of the Mn-S-state complex in the three types of TMF-2 preparations. The data show the rate constant measured at pH 6.5 is increased 4.5- and 12.5-fold in NaCl-TMF-2 and $CaCl_2$-TMF-2, respectively. Moreover, the 17 and 23 kDa proteins strongly affect the pH dependency (line 1 vs. 2). Since steric constraints do not limit access of NH_2OH to the cryptic Mn-S-state-enzyme, these observed differences can only reflect modifications of the reactivity of this enzyme with NH_2OH.

TABLE II

SECOND-ORDER RATE CONSTANTS FOR INACTIVATION OF Mn-S-STATE COMPLEX IN NaCl-, $CaCl_2$- AND TMF-2 PREPARATIONS

Type TMF-2 preparation	Second-order rate constant ($min^{-1} \cdot M^{-1}$)	
	pH 6.5	pH 7.5
TMF-2	80 [a]	160 [a]
NaCl-TMF-2	360 [a]	160 [a]
$CaCl_2$-TMF-2	1000 [b]	–

[a] Calculated from data of Fig. 6.

[b] Calculated from a 60 min extraction (Fig. 6 legend) with 5, 10 and 100 μM NH_2OH yielding 30, 43 and 94.2% solubilization of functional Mn, respectively. These Mn solubilizations were corrected for a 5% solubilization occurring in the absence of NH_2OH.

Discussion

Fig. 9 summarizes our results on the mechanism of NH_2OH inactivation of the Mn-polynuclear water oxidizing enzyme in the native state (TMF-2) and in NaCl- and $CaCl_2$-modified PS II membranes. Second-order rate constant values are indicated next to arrows. We postulate: (1) the native state exists as a supramolecular structure comprised of the tetra-Mn polynuclear complex, the PS II extrinsic proteins (17, 23 and 33 kDa) and possibly the 34 kDa [18] and other intrinsic polypeptides: and (2) the tetra-Mn polynuclear complex with Mn valency states of more than +2 [8,9] itself serves to stabilize structurally the complex yet function as an efficient catalyst of water oxidation. Fig. 9 attempts to illustrate our idea that the extrinsic proteins affect conformation of the complex either by provision of secondary ligands to the Mn-complex or by interactions (H-bonding, Van der Waals, electrostatic) within and between the intrinsic/extrinsic proteins comprising the structure. The first postulate is based on numerous reports indicating that steady-state V_{O_2} is diminished by extraction and/or perturbation of extrinsic proteins by NaCl and $CaCl_2$. By per-

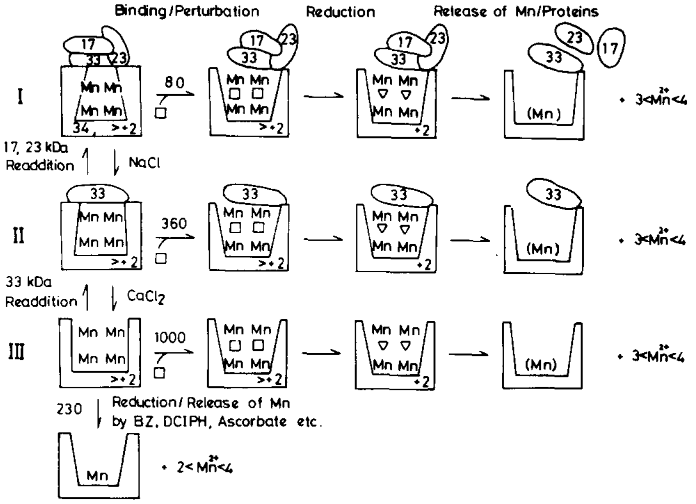

Fig. 9. Schematic model for the inactivation of the tetra-Mn supramolecular water-oxidizing enzyme by NH_2OH in TMF-2 (I), NaCl-TMF-2 (II) and by NH_2OH and weak redox reagents in $CaCl_2$-TMF-2 (III). □, NH_2OH: ▽, oxidized product of NH_2OH. Numbers by the arrows indicate the second order rate constant ($min^{-1} \cdot M^{-1}$) for Mn solubilization (or inactivation of V_{O_2}). The numbers in the right lower corners of the frames refer to valency state(s) of Mn in the tetra-Mn complex. Arguments for Model are given in Text.

turbation we imply modification of native structural conformation without solubilization of Mn or solubilization of protein. Such decreases in steady-state V_{O_2} are observed by modifications of Y_{O_2} patterns and in the oxidation of TMPD by PS II [44] following a single flash and also in Cl^- requirements for V_{O_2} (unpublished work in this laboratory).

The second postulate is based on the following arguments: (1) NH_2OH, NH_2NH_2 and their derivatives known to chemically reduce the S_1-state to S_0, or to form adducts with S_1, affect disassembly of the complex as judged by solubilization/perturbation of the ligated Mn and at least the extrinsic polypeptides: (2) the hierarchy of effectivity among such compounds for the Y_3 to Y_5 shift ($S_1 \rightarrow S_0$-NH_2OH [45]) in Y_{O_2} measurements vs disassembly of the native TMF-2 structure is qualitatively quite similar - $NH_2OH > CH_3$-$NHOH > NH_2OSO_3$, $NH_2NH_2 \gg CH_3NHOCH_3$ (Fig. 8): (3) though prior extraction of the 17 and 23 or the 17, 23 and 33 kDa proteins does not alter the observed hierarchy for inactivation of the Mn-complex, such extractions increase the chemical reactivity between NH_2OH and the Mn-complex and modify the pH dependency of the reaction (Fig, 6, Table II): moreover, reconstitution of V_{O_2} (NaCl-TMF-2) with the 17 and 23 kDa proteins results in characteristics of NH_2OH chemical reactivity very similar to the native TIMF-2 (Fig. 7): (4) in vitro rebinding (SDS-electrophoresis analyses) of the 17 and 23 kDa proteins occurs with NaCl-TMF-2, but not to NH_2OH preextracted NaCl-TMF-2 (unpublished results); and (5) in vivo reassembly of the 17 and 23 kDa proteins into the native-complex of NH_2OH extracted leaf segments occurs only during photoactivation of the S-state complex [46], the process in which Mn^{2+} becomes ligated possibly following photooxidation of Mn^{2+} by PS II to valency states more than +2 [16].

We therefore propose that the disassembly of the water-oxidizing complex structure and the Y_3–Y_5 shift in Y_{O_2} are similar mechanistically, both involving chemical reduction of higher Mn valencies in the S_1-state. In Fig. 9, the process of NH_2OH binding/perturbation is assumed to be rate-limiting. Our data argue that subsequent Mn reduction is primary in the disassembly process: however, we cannot totally exclude additional effects from NH_2OH in the disassembly process – for example, disturbance of the coordination sphere of Mn atoms within the tetra-Mn polynuclear complex, disruption of H-bonds and depletion of Ca from the PS II membranes. Indeed, we observe that 2 mM NH_2OH extraction of TMF-2 causes solubilization of approx. 1 Ca of approx. 2 Ca/200 Chl bound with high affinity (data not shown). If H-bond disruption by NH_2OH is involved, then 2 mM NH_2OH or less is far more effective than approx. 800 mM urea which we find to solubilize the 17 and 23 kDa proteins, but not the Mn from the complex. We are not convinced, however, from data in the literature [11–15] that distinct separation of the Y_3–Y_5 shift from the disassembly process has been shown.

Radmer and Ollinger [15] postulated that topographic constraints (approx. 0.4 nm wide and 0.25 nm deep) limited the access of bulky substituted polar amine derivatives to two Mn atoms of the S-state complex buried in a cleft in chloroplast membranes, based on their studies of the effectiveness of such compounds for affecting Y_3–Y_5 shift vs e^- donation to PS II in Tris-extracted chloroplasts. Applying their logic to our data, we are led to a similar conclusion even with PS II membranes devoid of the PS II extrinsic 17, 23 and 33 kDa polypeptides. On the other hand, Table II shows the removal of PS II extrinsic proteins increase the chemical reactivity between NH_2OH and Mn-S-state significantly. Furthermore, we found that lipophilic (benzidine (BZ), hydroquinone, $DCIPH_2$, TMPD) and even hydrophilic (ascorbate, ferrocyanide) redox reagents specifically cause Mn solubilization from $CaCl_2$-TMF-2, but not NaCl-TMF-2 (unpublished data). Similar observations have been reported by Ghanotakis et al. in their 2 M NaCl-extracted PS II membranes [47]. Therefore, these results lead us to the alternative explanation that the hierarchy of effectivity among polar amines for the inactivation of Mn-S-state enzyme may relate to the chemical reactivity of the polar amines and the Mn-S-state enzyme. In both cases redox reactivity appears to be the principal determinant.

Acknowledgments

We gratefully acknowledge support from USDA-SEA (82-CRCR-1-1062), NSF (PCM-11827) and DOE (AS05-83ER13072). This paper (85-3-45) is published with the approval of the Director of the Kentucky Agricultural Experimental Station. We thank Dr. D.F. Ghanotakis for valuable discussions and for bringing Ref. 46 to our attention. We also thank Carol Jean Gertsch for her expert technical assistance and Kirk Cammarata for providing the NaCl-TMF-2.

References

1 Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475.
2 Forbush, B., Kok, B. and McGloin, B. (1971) Photochem. Photobiol. 11, 307–321
3 Amesz, J. (1983) Biochim. Biophys. Acta 726, 1–12
4 Cheniae, G.M. and Martin, I.F. (1970) Biochim. Biophys. Acta 197, 219–239
5 Cheniae, G.M. and Martin, I.F. (1971) Plant Physiol. 47, 568–575
6 Golbeck, J.H., Martin, I.F. and Fowler, C.F. (1980) Plant Physiol. 65, 707–713
7 Yocum, C.F., Yerkes, C.T., Blankenship, R.E., Sharp, R.R. and Babcock, G.T. (1981) Proc. Natl. Acad. Sci. USA 78, 7507–7511
8 Wydrzynski, T. and Sauer, K. (1980) Biochim. Biophys. Acta 589, 56–70
9 Dismukes, G.C. and Siderer, Y., (1981) Proc. Natl. Acad. Sci. USA 78, 274–278
10 Dekker, J.P., Van Gorkum, H.J., Wensink, J. and Ouwehand, L. (1984) Biochim. Biophys. Acta 767, 1–9
11 Velthuys, B. and Kok, B. (1978) Biochim. Biophys. Acta 502, 211–221
12 Benoun, P. and Bouges, B. (1972) in Proceedings of the Second Congress on Photosynthesis Research, Stresa, 1971 (Forti, G., Avron, M. and Melandri, A., eds.), Vol. 1, pp. 569–576, Dr. W. Junk Publishers, Dordrecht, The Netherlands
13 Bouges, B. (1971) Biochim. Biophys. Acta 234, 103–112
14 Kok, B. and Velthuys, B. (1977) in Research in Photobiology (Castellani, A., ed.), pp. 111–119, Plenum Publishers, New York
15 Radmer, R. and Olligner, O. (1983) FEBS Lett. 152, 39–43
16 Radmer, R. and Cheniae, G. (1977) in Topics in Photosynthesis (Barber, J., ed.), Vol. 2, pp. 303–348, Elsevier Science Publishers, Amsterdam
17 Sharp, R.R. and Yocum, C.F. (1981) Biochim. Biophys. Acta 635, 90–104
18 Metz, J.G. and Bishop, N.I. (1980) FEBS Lett. 114, 61–66
19 Cammarata, K., Tamura, N., Sayre, R. and Cheniae, G. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 311–320, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, The Netherlands
20 Yamamoto, Y. and Nishimura, M. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Murata, N., Crofts, A.R., Renger, G., Govindjee and Satoh, K., eds.), pp. 229–238, Academic Press Japan, Tokyo
21 Kuwabara, T. and Murata, N. (1982) Plant and Cell Physiol. 23, 533–539

22 Ono, T.-A. and Inoue, Y. (1984) FEBS Lett. 164, 255–260

23 Sayre, R.T. and Cheniae, G.M. (1982) Plant Physiol. 69, 1084–1095

24 Toyoshima, Y. and Fukutaka, E. (1982) FEBS Lett. 150, 223–227

25 Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10

26 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234

27 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

28 Bradford, M. (1976) Anal. Biochem. 72, 248–254

29 Chua, N.H. (1980) Methods Enzymol. 69, 434–446

30 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) Biochim. Biophys. Acta 765, 388–398

31 Yocum, C.F., Sandusky, P.O., Ghanotakis, D. and Babcock, G.T. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 341–344, Martionus Nijhoff/ Dr. W. Junk Publishers, Dordrecht, The Netherlands

32 Den Haan, G.A., Duysens, L.N.M. and Egberts, D.J. (1974) Biochim. Biophys. Acta 368, 409–421

33 Smith, P.A.S. (1965) The Chemistry of Open-Chain Organic Nitrogen Compounds. W.A. Benjamin, Inc., New York

34 Dunahay, T.G., Staehlin, L.A., Seibert, M., Ogilvie, P.D. and Berg, S.P. (1984) Biochim. Biophys. Acta 764, 179–193

35 Ghanotakis, D.F. and Babcock, G.T. (1983) FEBS Lett. 152, 231–234

36 Reference deleted

37 Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87–93

38 Ljungberg, U., Jansson, C., Andersson, B. and Åkerlund, H.-E. (1983) Biochem. Biophys. Res. Commun. 113, 738–744

39 Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10

40 Raphaelian, L.A. (1966) in Encyclopedia of Chemical Technology (Kirk, R. and Othmer, D., eds.), pp. 164–196, Interscience, New York

41 Homann, P.H., Johnson, J.D. and Pfister, V.R. (1983) in the Oxygen Evolving system of Photosynthesis (Inoue, Y., Murata, N., Crofts, A.R., Renger, G., Govindjee, and Satoh, K., eds.), pp. 283–292, Academic Press Japan, Tokyo

42 Miyao, M. and Murata, N. (1984) FEBS Lett. 168, 118–120

43 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) FEBS Lett. 167, 127–130

44 Velthuys, B. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Murata, N., Crofts, A.R., Renger, G., Govindjee, and Satoh, K., eds.), pp. 83–90, Academic Press Japan, Tokyo

45 Radmer, R. and Ollinger, O. (1982) FEBS Lett. 144, 162–166

46 Callahan, F.E. (1984) Ph.D. Thesis, University of Kentucky

47 Ghanotakis, D.F., Topper, J.N. and Yocum, C.Y. (1984) Biochim. Biophys. Acta 767, 524–531