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Studies on the Reconstitution of O_2-Evolution of Chloroplasts[1]

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ABSTRACT

Extraction of spinach (*Spinacia oleracea* L.) chloroplasts with cholate-asolectin in the absence of Mg^{2+} results in the rapid and selective inactivation of O_2 evolution and a partial (30 to 40%) loss of photosystem II (PSII) donor activity without extraction of thylakoid bound Mn (~5 to 6 Mn per 400 Chlorophyll). Inclusion of ethylene glycol in the extractions inhibits loss of O_2 evolution and results in quantitative and qualitative differences in proteins solubilized but does not significantly inhibit the partial loss of PSII donor activity. Similarly, in two stage experiments (extraction followed by addition of organic solvent and solubilized thylakoid protein), O_2 evolution (V and V_{max}) of extracted chloroplasts is enhanced approximately 2.5- to 8-fold. However, PSII donor activity remains unaffected. This reversal of cholate inactivation of O_2 evolution can be induced by solvents including ethanol, methanol, 2-propanol, and dimethyl sulfoxide. Such enhancements of O_2 evolution specifically required cholate-solubilized proteins, which are insensitive to NH_2OH and are only moderately heat-labile. NH_2OH extraction of chloroplasts prior to cholate-asolectin extraction abolishes reconstitutability of O_2 evolution. Thus, the protein(s) affecting reconstitution is unlike those of the O_2 • Mn enzyme. The specific activity of the protein fraction effecting reconstitution of O_2 evolution is greatest in fractions depleted of the reported Mn-containing, 65-kilodalton, and the Fe-heme, 232-kilodalton (58-kilodalton monomer), proteins. Divalent (~3 millimolar) and monovalent (~30 millimolar) cations do not affect reconstitution of PSII donor activity but do affect reconstitution of O_2 evolution by decreasing the protein(s) concentration required for reconstitution of O_2 evolution in nonfractionated, cholate-asolectin extractions. The data indicate a reconstitution of the PSII segment linking the PSII secondary donor(s) to O_2-evolving centers.

Considerable information is available concerning the kinetic aspects of O_2 evolution (37) and the intermediates linking the S-states of O_2 evolution to P_{680} (6). Additionally, much evidence has accumulated which strongly suggests that thylakoid-bound Mn is essential in the O_2-evolving reactions (37) and that such Mn undergoes oxidation-reduction during cycling of the S-states (14, 40, 51).

By and large, however, this segment of the electron transport sequence of photosynthesis, in contrast to PSI, has been rather intractable to biochemical approaches aimed at the isolation and characterization of the proteinaceous catalysts involved (37). Such information is vital to the basic understanding of this biologically unique sequence of reactions, namely, the photosynthetic production of O_2.

Recently, two different approaches have been used in attempts to gain such insights: analyses of polypeptide composition and thylakoid Mn abundance of mutants blocked specifically on the oxidizing side of PSII (32, 33, 41); and detergent extraction and reconstitution analyses of chloroplasts with the use of artificial liposomes (35, 36, 44). Such studies have yielded disparate results. First, analyses of chloroplast membrane polypeptides of specific *Scenedesmus* mutants have led to the suggestion that a 34-kd polypeptide is probably associated with the Mn-requiring portion of the water-splitting apparatus of PSII (32, 33); however, the results of similar analyses of specific *Chlamydomonas* mutants (41) seem to question this conclusion. Second, extraction/reconstitution analyses have yielded evidence for the requirement of a 65-kd polypeptide in O_2 evolution which Spector and Winget (44) have identified as a Mn-protein and a site of Tris inhibition on the oxidizing side of PSII (37). This conclusion has been questioned, however, based on the observations that: (a) ≤1.9 Mn/400 Chl of the total thylakoid-bound Mn (~6 Mn/400 Chl) cosolubilized with the 65-kd polypeptide during cholate extraction of chloroplasts and that such solubilized Mn was dialyzable (41) and did not coprecipitate with the 65-kd polypeptide during ammonium sulfate precipitations (35, 41); and (b) a 58-kd monomer polypeptide, isolated by Nakatani and Barber (35, 36) employing essentially the procedures of Spector and Winget (44), proved identifiable with an Fe-heme but not with a Mn-protein but, nevertheless, yielded partial reconstitution of V_{O_2}[2] (35, 36) when incorporated into liposomes containing cholate-extracted chloroplasts (44).

Our inability (41) to reproduce the type extraction/reconstitution analyses as accomplished by others (35, 36, 44) has led us to explore other procedures which would permit extraction/reconstitution analyses, in an effort to clarify current data and possibly to obtain new information regarding specific protein requirements for the reactions on the oxidizing side of PSII. Here, we present evidence for a protein-dependent, organic solvent-induced reconstitution of O_2 evolution in cholate-asolectin-extracted chloroplasts. The results suggest a reconstitution of the sequence of reactions linking the O_2-evolving enzyme with secondary donors to P_{680}. Protein(s) required for the organic solvent-induced reconstitution of PSII electron transport have been partially purified and do not appear to be equivalent to those which reconstitute O_2 evolution in depleted photosomes (35, 44). (A preliminary report of these conclusions has been presented [41]).

MATERIALS AND METHODS

Chloroplast Preparation. Chloroplasts were prepared from market or greenhouse spinach (*Spinacia oleracea* L.) by the procedure

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[2] Abbreviations: V_{O_2}, rate of O_2 evolution; STM, 0.2 M sucrose, 0.02 M Na-Tricine (pH 8.0), 3 mM $MgCl_2$; ST, 0.2 M sucrose, 0.02 M Na-Tricine (pH 8.0); EG, ethylene glycol; FeCN, potassium ferricyanide; BQ, 1,4-benzoquinone; DPIP, 2,6-dichlorophenolindophenol; DPC, sym-diphenylcarbazide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; CMC, critical micelle concentration; Z_1, Z_2, chemically uncharacterized secondary electron donors to P680 of PSII; e, electron.

of Winget *et al.* (50), resuspended in STM buffer at ≥4 mg Chl/ml, and either used directly or stored at −80°C for up to 4 weeks until use. Such storage resulted in less than a 20% decrease in rates of O_2 evolution and no change in behaviors of extraction/reconstitution.

Cholate and Cholate-Asolectin Extraction. Chloroplasts in STM buffer were washed twice in 5 volumes of ST buffer, then resuspended at 4 mg Chl/ml in ST buffer prior to extraction with cholate or cholate-asolectin. Extractions were done at 4°C in darkness with stirring at 2 mg Chl/ml in ST buffer containing the designated concentrations of either recrystallized Na cholate or a mixture of Na cholate and asolectin liposomes. The Na cholate-asolectin liposomes were formed in small test tubes by sonication to clarity of a mixture of Na cholate and asolectin in ST buffer. Unless otherwise noted, the extraction duration was 40 min. Following extraction, the extracted chloroplasts were reconstituted without centrifugation or, alternately, following ultracentrifugation on a discontinuous sucrose gradient. The gradient, from bottom to top in tubes for the SW-40 Ti rotor (Beckman), consisted of 3, 4, and 2 ml of 1.8, 1.3, and 0.5 M sucrose-20 mM Tricine-NaOH (pH 8.0), respectively. Aliquots of the chloroplast extraction were layered, then centrifuged at 245,000*g* for 1 h. The Chl-containing bands, at the interfaces between the 1.3 and 0.5 M sucrose zones, were collected and subsequently used in reconstitutions.

Thylakoid Protein Extraction. Chloroplasts were extracted essentially as described in the preceding section, either with Na cholate (8 mg/ml) in ST buffer containing 100 mM NaCl or with preformed liposomes (8 mg/ml each of Na cholate and asolectin). Ultracentrifugation at 226,000*g* for 1 h yielded a yellowish supernatant and a firm pellet. Saturated $(NH_4)_2SO_4$ (pH 8.0) was added dropwise with stirring to the supernatant to increase the $(NH_4)_2SO_4$ concentration to 50%. After 30 min, the precipitate was collected by centrifugation at 20,000*g* for 10 min and redissolved in a small volume (protein ≥10 mg/ml) of 20 mM tricine-NaOH (pH 8.0). The protein fraction was dialyzed overnight at 4°C in pretreated (10 mM Na-EDTA [pH 7.5] at 100°C for 1 h) dialysis tubing *versus* 500 volumes of 20 mM Tricine-NaOH (pH 8.0), then stored at −80°C for periods of up to 4 weeks. This fraction was used for all reconstitution experiments unless otherwise noted.

Omission of 100 mM NaCl from the Na cholate extraction resulted in an unfirm pellet following ultracentrifugation and the formation of protein 'fluff,' largely at the liquid interface, rather than a pellet on subsequent centrifugation of the $(NH_4)_2SO_4$-precipitated proteins. Such behaviors were not observed in the extractions with cholate and asolectin.

Reconstitution. O_2 evolution of extracted chloroplasts or the thylakoid-containing bands from density gradient ultracentrifugation was reconstituted either by dialysis or by direct addition of the appropriate amount of designated solvent to the extracted chloroplast samples. Direct solvent-induced reconstitution was done simply by incubating cholate-asolectin-extracted chloroplasts in ST buffer (1 mg Chl/ml) containing designated solvents and other additions. The thylakoid-extracted proteins were added prior to the addition of solvents. Reconstitution was complete following a 45-min incubation at 4°C in the dark. With some batches of extracted chloroplasts subjected to fractionation by ultracentrifugation, it was necessary to include 1 mM $CaCl_2$ in both the extraction and reconstitution medium in order to reconstitute Hill activity (protein-dependent).

Reconstitutions were carried out also by dialysis of 1 ml extracted chloroplasts (2 mg Chl/ml) against 400 ml of ST buffer containing 25% (v/v) EG. Preliminary experiments showed that reconstitution of V_{O_2} by dialysis was complete within 2 h. Dialysis of unextracted control chloroplasts under identical conditions resulted in no more than 10% loss of V_{O_2}. Dialysis of extracted chloroplasts against ST buffer in the absence of EG yielded no change of V_{O_2}.

Rate Measurements of O_2 Evolution and Donor Photooxidation. Light was filtered through 2.54 cm of H_2O, and a Schott 116 and a Corning 3-69 filter then focused with suitable condensing lenses onto the vessel of 1.0-ml capacity. The light intensity, which was saturating for all assays, was varied where noted with neutral density filters. Rate measurements of O_2 evolution were made in a reaction mixture containing 1 mM FeCN and 30 mM methylamine in 0.4 M sucrose, 50 mM Tricine-NaOH (pH 7.5). Where indicated, BQ (2× sublimed) was used at 200 μM.

PSI donor photooxidations were made in a reaction mixture containing 100 μM methylviologen, 400 μM KCN, 30 mM methylamine, 5 mM ascorbate, 50 μM DPIP, and 10 μM DCMU in 0.4 M sucrose-50 mM Tricine-NaOH (pH 7.5). PSII donor photooxidations were made similarly, except for omissions of ascorbate, DPIP, and DCMU and inclusion of 5 mM NH_2OH or 500 μM DPC or 5 mM $MnCl_2$, as indicated. Chloroplast thylakoids equivalent to 10 to 15 μg Chl were used per assay.

Mn Determinations. All glassware and crucibles were washed in a 1:1 mixture of 1 N NHO_3 and 1 N HCl and rinsed with glass-distilled H_2O. Mn standards and chloroplast samples (50–200 μg Chl) were dried in porcelain crucibles at 80°C, then ashed at 500°C for 5 to 6 h. After cooling, 4 ml of 2 mM HNO_3 containing 5 mM HCl was added, and the covered crucibles were then heated at 50°C for 12 to 16 h. Sample volumes were adjusted to 4.0 ml with H_2O, then analyzed by automated flameless atomic absorption (Varian Model AA-175 equipped with Model CRA-90 and ASD-53) using 5 μl per analysis. Instrument settings were: wavelength, 279.46 nm; slit, 0.2 nm; dry, 100°C for 60 s; ash, 900°C for 30 s; atomize, 1,900°C for 3 s; and ramp rate, 600°C per s. By limiting the number of determinations per carbon rod to ≤60, less than 5% variation between duplicate analyses was obtained. Absorbance *versus* Mn concentration was linear up to at least 50 ng Mn/ml (yielding an absorbancy value of ~0.7).

SDS Gel Electrophoresis. SDS-Polyacrylamide gel electrophoresis, protein solubilization in the presence of SDS, and polypeptide visualization was done as described by Chua (11), with the exception that a 12-cm, 7.5 to 15% linear polyacrylamide gradient was used. Equivalent amounts of proteins were applied per slot.

Protein and Chl Determinations. Proteins were determined by the method of Lowry *et al.* (30) with deoxycholate (0.1% [w/v]), and Chl was determined by the method of Arnon (3).

Chemicals. Cholic acid (Sigma Chemical Co.) was purified and recrystallized (2×) as described by Spector and Winget (44). Asolectin (Associated Concentrates, Woodside, NY) was extracted of acetone- and ether-insoluble materials (44) and stored in $CHCl_3$ solution (100 mg asolectin per ml) under N_2 at −15°C. 'Sequanal' grade $(NH_4)_2SO_4$ (Pierce Chemical Co.) was used, and all other chemicals were reagent grade.

RESULTS

Cholate Inactivity of Photosynthetic Electron Transport and Its Inhibition by EG. The data of Figure 1A describe the time courses of loss of V_{O_2} (curve 2), PSII donor photooxidation (curve 3), and PSI donor photooxidation (curve 4) of chloroplasts (2 mg Chl/ml) subjected to extraction with Na cholate and asolectin (8 mg/ml each) in STM buffer. Curve 1 shows V_{O_2} of control chloroplasts. All measurements employed methylviologen as the electron acceptor. The data show that extraction for only 30 s resulted in about 80, 30, and 35% decreases in V_{O_2}, PSII, and PSI donor photooxidation, respectively. With increasing duration of extraction, both PSII and PSI activities decreased another 20 to 25% over the course of 50-min extraction. The results, obtained in the presence of STM buffer, suggest rather rapid (≤30 s) effects of cholate-asolectin extraction on V_{O_2} as well as on PSI and PSII partial reactions.

The data of Figure 1B were obtained similarly to those of

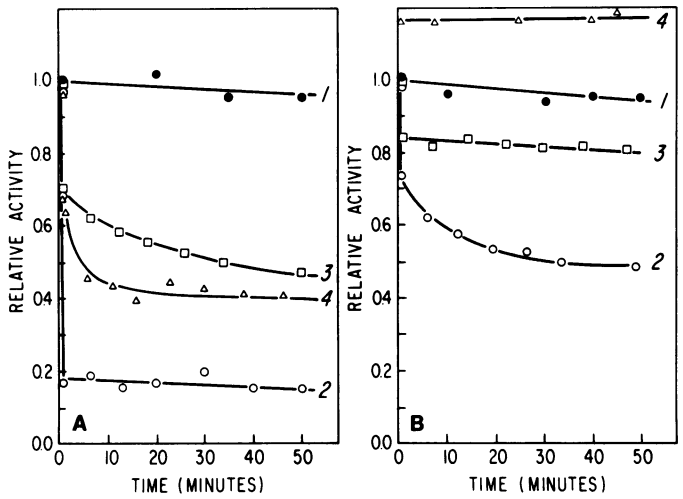

FIG. 1. Time course of inactivation of O_2 evolution and PSII and PSI donor activities by cholate-asolectin extraction in the absence (Fig. 1A) and presence of 25% (v/v) EG (Fig. 1B). Chloroplasts (2 mg Chl/ml) were incubated in STM containing 8 mg/ml each of Na cholate and asolectin for the times indicated on the abscissa; 7.5 μl of the suspension were then injected into the appropriate assay medium ("Materials and Methods") within the polarographic assay vessel. Curve 1, V_{O_2} of control; curve 2, V_{O_2} of cholate-asolectin-extracted chloroplasts; curve 3, PSII donor activity measured at quantum yield intensity; curve 4, PSI donor activity. The initial absolute values for V_{O_2} and PSI and PSII donor activities were 1432, 1708, and 155 eq/Chl·h, respectively. Methylviologen was the electron acceptor in all assays.

Figure 1A, except for the inclusion of 25% (v/v) of EG. This solvent effectively stabilizes activities of chloroplasts during storage at low temperature (16). It also causes degeneration of cholate micelles (31), can solubilize hydrophobic membrane proteins (28), and is believed to increase H-bonding and electrostatic interactions, relative to H_2O, while decreasing solvophobic interactions (17, 18, 20, 22–24).

Comparison of the data of Figure 1B with those of Figure 1A shows that inclusion of EG in the extraction medium caused significant inhibitions (40 and 62%, respectively, measured at 50 min) of loss of V_{O_2} and PSII donor activities while enhancing PSI donor activity by about 17% beyond levels of the control. A portion of the loss of PSII donor activity (Fig. 1A, curve 3), as we measured it, conceivably might be confounded, because of simultaneous loss of PSI donor activity, even though we assayed at conditions permitting interchain e transport (43). Such criticism does not appear to apply in the data of Figure 1B, from which we tentatively conclude that, although addition of EG abolishes apparent cholate-asolectin-induced inactivation of PSI donor activity, significant extents of inactivation of O_2 evolution and PSII donor activity still occur. In later sections, we present evidence that suggests an EG-dependent reconstitution of that segment of the e transport sequence that links the O_2-evolving enzyme to PSII traps. Data such as Figure 1B, therefore, pertain to the extents of reconstitution obtainable in the extraction-reconstitution system used in later sections.

The data of Table I further analyze the effects of EG on cholate-asolectin-induced loss of O_2 evolution and partial reactions of the electron transport chain as well as the effects of 3 mM $MgCl_2$ during extraction on these activities. We noted the following. First, in the absence of Mg^{2+} and EG, no decrease of PSI donor photooxidation (DCIPH$_2$-viologen) was observed, and the V_{O_2} (viologen) and PSII donor photooxidation measured through viologen was approximately 2-fold greater than that obtained with extraction in the presence of Mg^{2+}. Second, the extents of loss of V_{O_2} (viologen *versus* FeCN) in the absence of EG were similar only in extractions without Mg^{2+}. Third, in the presence of EG, the extents of losses of V_{O_2} were entirely similar and independent of Mg^{2+}, regardless of electron acceptor, reflecting the lack of loss of PSI donor photooxidation in the presence of EG. Fourth, in the presence of EG, addition of Mg^{2+} to the extraction mixture diminished, but did not totally exclude, apparent inactivation of PSII donor activity.

The results of Table I indicate that omission of Mg^{2+} from the extraction medium restricted the effects of cholate-asolectin extraction to PSII but increased slightly (~15–18%) the extent of inactivation of PSII donor activity observed in the presence of EG. However, the presence or absence of Mg^{2+} did not affect the rapid phase of inactivation of O_2 evolution. Over the course of many such experiments with many different chloroplast preparations, the extents of inactivation of O_2 evolution and PSII donor activity by extraction (30–60 min) with cholate-asolectin in the absence of Mg^{2+} was usually 80 to 90% and 50 to 60%, respectively. Unless otherwise noted, Mg^{2+} was omitted from the extractions in all subsequent experiments.

Data such as those of Table I also were obtained with extracted and ultracentrifuged thylakoids, thus permitting comparisons with similar data (44) obtained at different extraction conditions; namely, a high cholate:Chl ratio (10:1) in the presence of 3 mM Mg^{2+} and 400 mM $(NH_4)_2SO_4$. Such extraction resulted in total loss of O_2 evolution as well as PSII and PSI donor activities (44). It seems clear, however, that similar high concentrations of cholate or deoxycholate do not abolish either P680 or P700 absorbance changes (39, 48).

Solubilization of Chloroplast Membrane Components by Cholate-Asolectin. In contrast to many other detergents which, like cholate, also inactivate O_2 evolution, low concentrations of cholate (<15 mg/ml) do not solubilize Chl and solubilize only a small amount of the Cyt or other thylakoid proteins and lipids (7). Recently, attention has been focused on the cholate-solubilized polypeptides, 65- (21, 44) and 58-kd monomers (35, 36), as proteins specifically required in O_2 evolution. We (40), as well as Nakatani and Barber (35, 36), have questioned the assignment of the 65 kd polypeptide as a Mn-containing protein.

The capacity of EG to inhibit or prevent significantly the inactivation of O_2 evolution by cholate-asolectin (or cholate alone) (Fig. 1; Table I) suggested that quantitative and qualitative differences might exist in solubilized proteins obtained from extractions in the presence and absence of EG. The data of Table II compare the effect of cholate-asolectin extraction in the presence and absence of EG on V_{O_2}, thylakoid Mn abundance, and solubilization of thylakoid proteins, while lanes 4 and 5 of Figure 2 show SDS-PAGE analyses of the solubilized supernatant proteins from the two types of extractions.

From Table II, we note the following. First, in the absence of EG, over 90% of O_2 evolution was abolished in either the extracted suspension or the 144,000*g* pellet, but less than 8% (0.42 Mn/400 Chl) of the Mn pools considered to be required for O_2 evolution and possible PSII function (37) was extracted. Additionally, 14% of the total thylakoid protein was solubilized. Second, in the presence of EG, the rates of O_2 evolution of the extracted chloroplasts (extracted suspension or 144,000*g* pellet) were approximately 10-fold greater than were the same fractions from extractions in the absence of EG and were 71% of the original unextracted suspension. Additionally, the presence of EG in the extraction diminished the amount of protein solubilized by 43% such that only 8% of the total thylakoid protein was solubilized.

Lanes 4 and 5 of Figure 2 show SDS-PAGE polypeptide profiles of thylakoid proteins precipitated by 2 M $(NH_4)_2SO_4$ that had been solubilized by cholate-asolectin in the absence and presence, respectively, of 25% (v/v) EG. The proteins solubilized by cholate-asolectin in the presence of EG were first dialyzed to remove EG (and cholate) before readdition of cholate to the original concen-

Table I. *Effect of Extraction Conditions on Cholate-Asolectin-Induced Loss of V_{O_2}, PSI, and PSII Activities*

Reaction Assayed	Extraction Duration	Extraction Condition			
		Plus Mg^{2+}		Minus Mg^{2+}	
		Minus EG	Plus EG	Minus EG	Plus EG
	min	*relative activity*[a]			
H_2O-viologen	30	0.17	0.80	0.39	0.85
H_2O-FeCN[b]	30	0.36	0.72	0.34	0.74
NH_2OH-viologen	30	0.33	0.86	0.63	0.71
$DCIPH_2$-viologen	30	0.39	1.12	1.18	1.20
H_2O-viologen	60	0.12	0.75	0.33	0.74
H_2O-FeCN	60	0.35	0.69	0.25	0.71
NH_2OH-viologen	60	0.30	0.87	0.57	0.69
$DCIPH_2$-viologen	60	0.34	1.06	1.07	1.15

[a] Absolute values, expressed in eq/Chl·h, of controls for first, second, third, and fourth reactions were 1288, 1212, 149, and 1780, respectively. The results represent averages of three separate experiments with the same batch of chloroplasts. For other details, see "Materials and Methods."
[b] Addition of BQ (200 μM) to the assay mixture did not change the relative values shown but did increase the absolute values by 10 to 15%.

Table II. *Effect of Cholate/Asolectin Extraction in the Presence and Absence of EG on V_{O_2}, Thylakoid Mn Abundance, and Solubilization of Thylakoid Proteins*

V_{O_2} was determined with FeCN. Extraction in ST buffer was for 40 min. For other details, see "Materials and Methods."

Chloroplast Fraction	V_{O_2}		Mn abundance,	Protein Concentration	
	−EG	+EG	−EG	−EG	+EG
	O_2/Chl·h		*Mn/400 Chl*	*mg/ml*	
Original suspension	304	295	5.41	18.0	18.0
Extracted suspension	23	210	5.33	18.0	18.0
144,000*g* Pellet	17	211	4.95		
144,000*g* Supernatant				2.52	1.44
STM-washed, 144,000*g* Pellet[a]	9		2.60		
NH_2OH-washed, 144,000*g* Pellet[a]	0		2.16		

[a] The 144,000*g* pellet from the extraction was resuspended and homogenized in STM buffer. Washes were made by incubating the thylakoids (200 μg Chl/ml) for 10 min in STM buffer containing 5 mM NH_2OH, where noted, then centrifuging to recover the pellet. Pellets were washed once with STM buffer before resuspension and determinations of V_{O_2} and Mn.

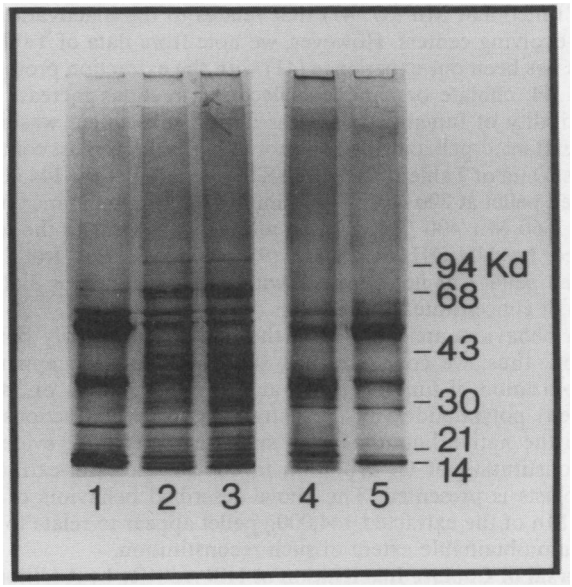

FIG. 2. SDS-PAGE profiles of chloroplast polypeptides solubilized by cholate-$(NH_4)_2SO_4$ (35, 44) or by cholate-asolectin and precipitated by $(NH_4)_2SO_4$. Lanes 1 through 3 represent solubilized chloroplast polypeptides from cholate-$(NH_4)_2SO_4$ extractions, according to the procedures of Spector and Winget (44) and Nakatani and Barber (35) and precipitated by 2, 1.2, and 0.84 M $(NH_4)_2SO_4$, respectively. Lanes 4 and 5 are solubilized chloroplast polypeptides from cholate-asolectin extractions carried out in the absence or presence of 25% (v/v) EG, respectively, and precipitated by 2 M $(NH_4)_2SO_4$. Equivalent amounts (50 μg) of protein were applied to each lane (see text for details).

tration and subsequent $(NH_4)_2SO_4$ precipitation. Accordingly, the profiles obtained from equivalent amounts (50 μg protein) of applied protein permit meaningful analyses for any qualitative differences in the proteins solubilized by the two extraction conditions.

Shown also in Figure 2 for comparative purposes are polypeptide profiles of thylakoid proteins solubilized by cholate (cholate/Chl of 10 [mg/mg]) in the presence of 0.4 M $(NH_4)_2SO_4$, according to procedures of Spector and Winget (44) and Nakatani and Barber (35, 36), and precipitated by 2.0, 1.2, and 0.84 M $(NH_4)_2SO_4$ (lanes 1, 2, and 3, respectively). The profiles in lanes 1, 2, and 3 show the extraction and subsequent enrichment by $(NH_4)_2SO_4$ precipitation of the (65- and 58-kd polypeptides, in agreement with the observations of Spector and Winget (44) and Nakatani and Barber (35, 36). In no instance, however, have we obtained a 65-kd fraction containing Mn (41). Thus, on this point, our results, as well as those of Nakatani and Barber (35, 36), disagree with those of Spector and Winget (44).

A comparison of the polypeptide profiles of lanes 1 through 3 *versus* the polypeptide profiles of the proteins extracted and precipitated by conditions used here (lane 4) shows clearly that our conditions result in minimal solubilization and enrichment of either the 65- or the 58-kd polypeptides. A comparison of the polypeptide profiles of lane 4 *versus* lane 5 (extraction without and with EG, respectively) shows that EG effectively minimizes the cholate-asolectin-induced solubilization of at least six polypeptides ranging in mol wt from ~10 to 35 kd, most predominantly polypeptides in the regions of 19, 21 to 23, and 34 to 36 kd, but that extraction in the presence of EG results in apparent increased solubilization of polypeptides in the regions of ~14 to 16, as well

as 44 to 49, kd. The polypeptides in this latter region may be associated with the reaction center II Chl protein(s) (10, 34). If we accept this identification, we argue that the amount of these polypeptides solubilized in the presence of EG represents only a small fraction of the total abundance in thylakoids based on the following evidence: V_{O_2} of thylakoids extracted in the presence of EG are 70% of controls (Table II); and SDS-PAGE analyses of pellets of such extractions revealed no discernible deletions of polypeptides in this region.

Clearly, the data of Table II and Figure 2 indicate that the EG protection against cholate-asolectin-induced loss of O_2 evolution is accompanied by both quantitative and qualitative differences in solubilization of polypeptides but without significant solubilization of functional Mn. Here, we note absence of cosolubilization of functional Mn (Table II) and polypeptides in the 34- to 36-kd region (Fig. 2, lane 4). Thus, we question the tentative assignment of a 34-kd polypeptide as a polypeptide derived from a Mn-binding protein associated with the water-splitting apparatus of PSII (32, 33).

The data here and elsewhere (41) on solubilization of functional Mn by cholate (44) or deoxycholate (2) suggest that, unlike other treatments specifically abolishing V_{O_2} (37), there is no apparent loss of functional Mn (37, 47) that relates to the inactivation of the O_2-evolving centers. However, we note from data of Table II that, as has been our experience (41) with the extraction protocols of Ref. 44, cholate or cholate-asolectin extractions increase the susceptibility of functional Mn loss during subsequent washings of the extracted pellet at low, but not at high, chloroplast concentrations. Data of Table II show that STM washing of the 144,000*g*-extracted pellet at 200 μg Chl/ml diminished Mn abundance from 4.95 to 2.60 Mn/400 Chl, a value almost equivalent to the level obtained by NH_2OH extraction (9). Such Mn loss from the extracted pellet was not observed with STM washings or dialysis (4–6 h) of concentrated suspensions (4 mg/ml).

Such behaviors are atypical of the native functionally bound Mn, and, thus, we conclude that, though there is no apparent cosolubilization of functional Mn and of either the 65- or 34-kd (assumed) polypeptides by the extractions, such extractions do perturb the native bound Mn. In subsequent sections, evidence for reconstitution of O_2 evolution in cholate-asolectin-extracted chloroplasts is presented. The above-described behaviors of the bound Mn of the extracted 144,000*g* pellet appear to relate to the maximum obtainable extent of such reconstitution.

Reversal of Cholate Inactivation of Hill Activity by Addition of Organic Solvents. The inhibition by EG of loss of O_2 evolution and, to a lesser extent, PSII donor activity by extraction with cholate-asolectin in ST buffer conceivably could reflect either simple inhibition of the solubilization of proteins essential for the oxidizing side of PSII, as implied by the data of Tables I and II and Figure 2, or a dissociation by EG of thylakoid-bound cholate, which rather specifically inhibits O_2 evolution. This latter supposition is based on observed rapid (≤30s) inhibition or abolishment of V_{O_2} (41), the extent dependent on cholate-to-Chl ratios (data not shown) and the literature indicating avid binding of cholate to many membrane proteins (24, 25, 28).

The first hypothesis implies that any EG-induced increase of V_{O_2} of cholate-asolectin-extracted chloroplasts would be strictly dependent on added thylakoid-solubilized protein and would involve an EG-induced reinsertion of cholate-solubilized protein(s) into the extracted thylakoid membrane, *i.e.* reconstitution. The second hypothesis was tested by various procedures to remove presumed inhibitory-bound cholate from uncentrifuged, extracted thylakoids. The procedures included dilution (≤100-fold) or dialysis (≤24 h) *versus* ST buffer and passage over Biogel A 1.5 M exclusion gels. Neither of these procedures resulted in any increase of V_{O_2} of the extracted thylakoid suspensions.

However, the addition of EG to uncentrifuged, extracted chloroplast suspensions containing cholate-asolectin-solubilized proteins did result in a significant increase of V_{O_2} (Table III; Fig. 3) in these two-stage experiments. The data of Table II show that: (a) a concentration of EG effective in eliciting an ~2-fold increase in V_{O_2} of extracted, uncentrifuged chloroplasts had no effect on V_{O_2} or on PSII and PSI donor activity of control chloroplasts; and (b) the diminished rates of PSII, but not of PSI, donor activity, resulting from the extraction, were unchanged after addition and incubation with EG. The results of Table III, thus, are entirely in agreement with the data of Figure 1B and Tables I and II, except for differences in the magnitude of the effect(s) of EG on protection *versus* reactivation of O_2 evolution. In both cases, we deal with the oxidizing side of PSII and, more specifically, the region beyond the site of NH_2OH entry into PSII. Over the course of a year of similar type experiments involving many different chloroplast preparations, we observed up to 8-fold increases of V_{O_2} in extracted, uncentrifuged chloroplasts, corresponding to 55% of the control chloroplasts, on supplementation with solubilized thyla-

Table III. *Inactivation of Photosynthetic Electron Transport by Cholate-Asolectin Extraction and Partial Reactivation of V_{O_2} Specifically by EG*

See "Materials and Methods" for extraction, reactivation, and assay procedures.

Reaction Assay	Control Rate (Plus or Minus EG)	Extracted Rate: Plus ST	Extracted Rate: Plus EG
	eq/Chl·h		
$H_2O \rightarrow$ BQ/FeCN	1,304	128	284
$H_2O \rightarrow$ viologen	1,208	124	208
$NH_2OH \rightarrow$ viologen	480	210	202
DPC → viologen		210	224
$DCIPH_2 \rightarrow$ viologen + DCMU	1,542	1,544	1,528

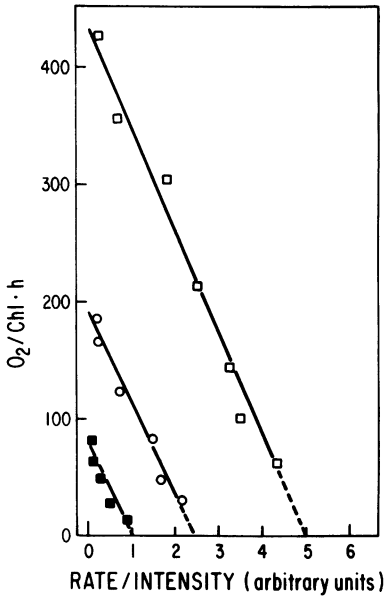

FIG. 3. O_2 rate *versus* rate/intensity plot of control, cholate-asolectin-extracted, and EG-reactivated chloroplasts. The rate/intensity intercept is the apparent quantum yield for Hill activity, and the (−) slope equals the rate constant. ■—■, Extracted chloroplasts; O—O EG-reactivated chloroplasts; and □—□, control chloroplasts. Chloroplasts were prepared according to procedures described in "Materials and Methods." Cholate-extracted chloroplast proteins (protein:Chl ratio [g/g], 1:1) were added before EG (25% [v/v]) to the reactivated chloroplasts.

koid protein fractions. Never have we observed a lack of increased rates of O_2 evolution from addition of EG to extracted chloroplasts.

The effectiveness of EG in restoring the 30 to 50% loss of PSII donor photooxidation under our extraction conditions ($-Mg^{2+}$; Tables I and II) implies an upper limitation of the EG effects on V_{O_2}, since O_2 centers behave as independent units (37). However, this argument is valid only if we deal with a reactivation of V_{O_2} and not with relief of a rate limitation imposed by extraction. Figure 3 shows rate *versus* intensity relationship for V_{O_2} of control, extracted, and reactivated (+EG) chloroplasts. The data show that the extraction causes a marked drop of the quantum efficiency of V_{O_2} (intercept on abscissa) but is partially reversed (2.5-fold increase) following addition of EG and supplemental, solubilized thylakoid proteins to the extracted chloroplasts. Similar effects on V_{max} (intercept on ordinate) were observed. The data of Table III and Figure 3, thus, indicate that we deal with an EG-induced reactivation of electron flow between the presumed intact Mn-containing, S-state complex and the PSII traps retaining capacity for NH_2OH photooxidation following extraction.

Figure 4 shows that several organic solvents other than EG also partially reversed, in the two-stage experiment, the cholate-asolectin inactivation of V_{O_2}. These data show ~3-fold maximum increase of V_{O_2} of cholate-asolectin uncentrifuged, extracted chloroplasts on addition of the appropriate concentrations of 2-propanol, ethanol, or methanol. Additionally, DMSO (35% [v/v]), but not glycerol (0 to 50% [v/v]), proved effective. The magnitude of increase of V_{O_2} with optimum concentrations of such solvents proved equivalent to that obtainable with EG. However, in contrast to EG, which could be used at concentrations as high as 50% (v/v) without inactivation of control or extracted chloroplasts, the other solvents, particularly 2-propanol, at high concentrations yielded inactivation of both control and extracted chloroplasts (Fig. 4). Accordingly, EG was used in all subsequent work.

Protein Dependent Reconstitution of V_{O_2} by EG. The ineffectiveness of simple dilution, dialysis *versus* ST buffer, or gel filtration for the reactivation of V_{O_2} of uncentrifuged, extracted chloroplasts possibly argues against the second hypothesis—namely, that reactivation in the two-stage experiments reflects a dissociation of bound inhibitory cholate. However, the routine, consistent, and specific reactivation of V_{O_2} on addition of organic solvents could be a consequence of a simple solvent-induced increase of the dissociation of the presumed inhibitory-bound cholate rather than of the solvent-induced reinsertion of protein(s) into the thylakoid membrane (first hypothesis). The first hypothesis dictates that any observed reactivation (reconstitution) must show strict dependence, specificity, and saturation behavior for the protein(s). On the other hand, the second hypothesis implies independence of solubilized protein in the solvent-induced reactivation.

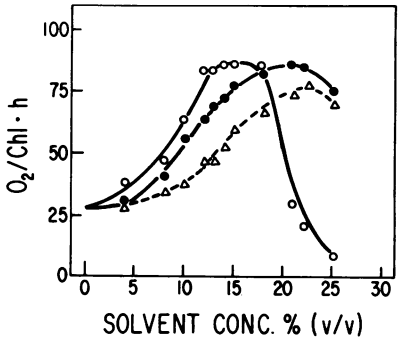

FIG. 4. Reconstitution of O_2 evolution in cholate-asolectin-extracted chloroplasts by addition of organic solvents. 2-Propanol (○——○), ethanol (●——●), and methanol (△——△) were added as described in "Materials and Methods." Control rates of O_2 evolution were 280, 272, 250, and 94 O_2/Chl·h, with no additions, and plus 22% (v/v) methanol, ethanol, and propanol, respectively.

Figure 5 shows the effect(s) on V_{O_2} of addition of BSA and dialyzed supernatant proteins (226,000g/60 min) to uncentrifuged, cholate-asolectin-extracted chloroplasts in the presence or absence of 3 M EG. We noted that, in the absence of EG, incubation (60 min) of extracted, uncentrifuged chloroplasts with increasing amounts of cholate-asolectin-solubilized thylakoid proteins yielded no reactivation of V_{O_2}; however, in the presence of EG, increasing amounts of thylakoid-solubilized proteins resulted in increasing V_{O_2}, until a maximum was reached at 300 μg added protein (protein/Chl ratio of 0.75 on a mg basis). Assuming, for the moment, that the increase of V_{O_2} observed on addition of EG alone to uncentrifuged, extracted chloroplasts represents an effect from solubilized protein in the extraction milieu, then the maximum observable protein effect on V_{O_2} in the data of Figure 5 is 3.5-fold; concentrations of BSA equivalent to or 8-fold greater than added solubilized thylakoid proteins did not effect V_{O_2} in the presence of EG. In similar experiments (data not shown), with quantum yield measurements of PSII photooxidation of NH_2OH (26, 37) no effects were observed from incubations of uncentrifuged, extracted thylakoids with solubilized thylakoid proteins either in the presence or in the absence of EG. These data argue for an EG-induced specific increase of V_{O_2}, which is dependent on specific thylakoid solubilized proteins which are saturable. The data, therefore, support the first hypothesis and argue for a protein-dependent, EG-induced reconstitution of the reaction sequence linking the PSII entry site of NH_2OH to the O_2-yielding reactions.

This argument is further supported by the data of Table IV. Here, we determined the extents of reconstitution obtainable by the addition of EG or EG plus a cholate-solubilized, $(NH_4)_2SO_4$-

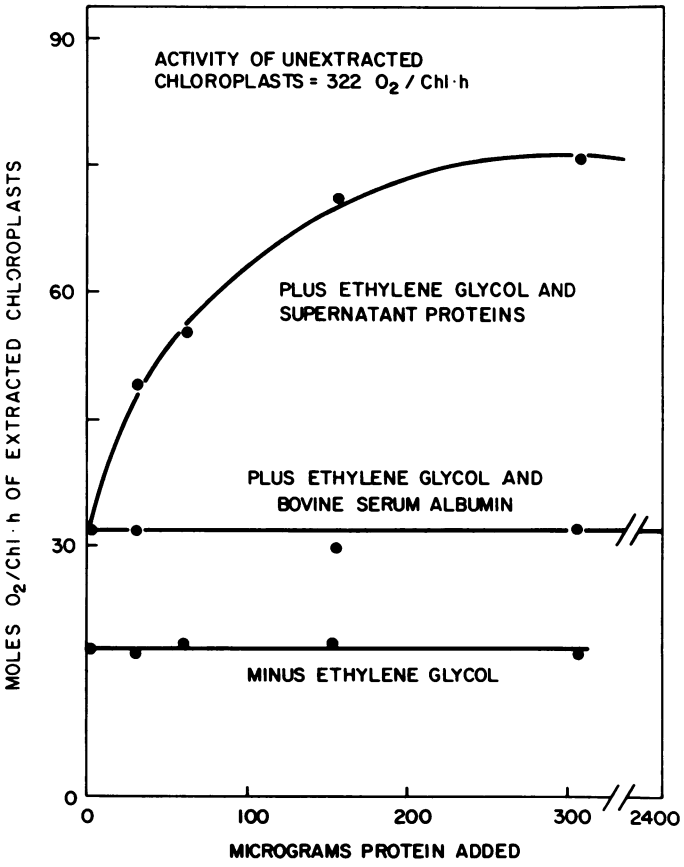

FIG. 5. Reconstitution of cholate-asolectin-extracted chloroplasts requires supernatant protein(s). See text for details.

Table IV. *Reconstitution of O_2 Evolution in Nonfractionated and Fractionated (Ultracentrifuged) Cholate-Asolectin-Extracted Chloroplasts Requiring Thylakoid-Solubilized Proteins*

Control rates were 295 and 209 O_2/Chl·h for experiments 1 and 2, respectively. Extraction, fractionation, and reconstitution procedures are described in "Materials and Methods."

Addition	Extracted and Nonfractionated Chloroplasts		Extracted Chloroplast Membrane Fraction	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
	O_2/Chl·h			
ST buffer	69	39	64	44
ST buffer plus EG	96	70	63	44
ST buffer plus EG plus extracted thylakoid proteins	115	94	104	83

precipitated thylakoid protein fraction to uncentrifuged, extracted chloroplasts or to cholate-asolectin-extracted chloroplasts subjected to centrifugation on a discontinuous gradient to separate the extracted proteins from the depleted membrane. For unknown reasons, we experience considerable variability in obtaining reconstitution under any conditions with ultracentrifuged pellets derived from extractions. This may reflect requirements of thylakoid membrane structure which is disturbed by pelleting and resuspension (see "Materials and Methods"). Accordingly, in the experiments of Table IV, we used density-gradient centrifugation to separate the extracted thylakoid membranes from solubilized proteins.

Though the magnitudes of reconstitution shown in Table IV are less than those presented in earlier sections, it seems clear that any observed increased rate of O_2 evolution is dependent on the presence of solubilized thylakoid proteins during incubation of extracted membranes with EG. Since no increased rate of O_2 evolution was ever observed on incubation with EG of thylakoid membranes devoid of solubilized proteins, it seems unlikely that the EG-induced increased rates of O_2 evolution of the cholate-asolectin-extracted chloroplasts are a consequence of dissociation of membrane-bound, inhibitory cholate. The results of Figure 5 and Table IV, therefore, argue for a solvent-induced reconstitution of cholate-solubilized proteins with the extracted chloroplast membrane.

Effects of Cholate-Asolectin Liposomes on the Inactivation and EG-Induced Reconstitution of V_{O_2}. Data in previous sections were obtained by extractions of chloroplasts with preformed, cholate-asolectin liposomes at 4 mg each of asolectin and cholate per mg Chl. The data in Figure 6 (A and B) explain this rationale and yield information on the importance of the liposomes for the determination of direct reconstitution of V_{O_2} of extracted chloroplasts.

Curve 1 of Figure 6A illustrates the effect on V_{O_2} of incubation of chloroplasts for 40 min at the designated cholate-to-Chl ratios given on the abscissa. The data show a 71% loss of V_{O_2} at a cholate:Chl ratio of 4, with no significant additional loss of V_{O_2} with increasing cholate-to-Chl values. Curve 2 was obtained similarly, with the inclusion of asolectin (4 mg asolectin/mg Chl) as preformed liposomes. We noted that the inclusion of asolectin liposomes substantially increased the extent (by ~24%) of inactivation relative to that obtained with cholate alone at cholate:Chl ratios of >3:1. Asolectin addition to control chloroplasts had no effect on V_{O_2}, nor were rates of O_2 evolution of cholate-asolectin-extracted chloroplasts affected by additions of BSA (1 mg/ml). Thus, the effects of asolectin liposomes on V_{O_2} were not due to contaminating free long-chain fatty acids (19). Curve 3 was obtained similarly to curves 1 and 2, except that asolectin concentration (as liposomes) was varied at a constant cholate:Chl ratio (4:1). These data reinforce conclusions obtained from curve 2 and show an enhancement of inactivation of V_{O_2} by asolectin in the presence of cholate (cholate:Chl ratio of 4:1) and saturation of the effect at an asolectin:Chl ratio of 4:1.

Figure 6B shows V_{O_2} following EG-induced reconstitution in the presence and absence of supplemental, solubilized thylakoid protein(s) of the chloroplasts extracted at the various conditions of Figure 6A. Curve 5 shows V_{O_2} of reconstituted curve 1 chloroplasts (alone cholate extraction) with and without supplemental, solubilized thylakoid proteins. We noted an increase of V_{O_2} from reconstitution of ~1.8-fold with chloroplasts extracted at cholate:Chl ratios of ≥4:1 and a reconstituted V_{O_2} equivalent to ~52% of the control (unextracted) chloroplasts. We also noted that supplemental protein had no effect on the extents of reconstitution.

Curve 4 and curve 6 show V_{O_2} of reconstituted curve 2 chloroplasts (cholate-asolectin liposome extractions) in the absence and presence, respectively, of supplemental, solubilized thylakoid proteins. We noted an ~6-fold increase of V_{O_2} by reconstitution of chloroplasts extracted with cholate:Chl ratios >4:1 in the presence of asolectin liposomes (asolectin:Chl ratio of 4:1) and in the absence of supplemental proteins. Such reconstituted V_{O_2} values were ~29% of control (unextracted) chloroplasts. The addition of supplemental, thylakoid-derived proteins to the curve 2, extracted chloroplasts yielded an additional increase (~1.8-fold) of V_{O_2} such that the total increase of V_{O_2} from reconstitution was ~10-fold, equivalent to ~52% of control (unextracted) chloroplasts and equivalent to reconstituted curve 1 chloroplasts (cholate extraction alone).

Clearly, the data of Figure 6 (A and B) indicate that, in the presence of cholate at concentrations greater than the (cholate) CMC (Fig. 6A), asolectin liposomes increased the extent of inactivation of V_{O_2} as well as limited the magnitude of the reconstitution of V_{O_2} induced by EG (Fig. 6B). Reconstitutions of cholate-asolectin-extracted chloroplasts responded to the addition of solubilized thylakoid proteins, maximizing the extent of reconstitution.

We noted that extraction with liposomes (cholate:asolectin ratio of 1:1 and cholate:Chl ratio of 4:1) yields no quantitative difference in proteins solubilized by cholate alone at equivalent concentrations but that qualitative differences exist in the proteins solubilized, as revealed by SDS-PAGE analyses. Preliminary results indicated that solubilization of polypeptides in the 20-kd and 35-kd regions was most effected by the inclusion of asolectin liposomes in the extraction medium. We interpret the effects of cholate-asolectin liposomes on extraction and subsequent reconstitution as possibly involving an increased solubilization, compared to cholate alone, of specific polypeptides essential for V_{O_2} and an increased 'sequestering' or binding of the solubilized proteins relative to cholate micelles, such that any quasiequilibrium state between the solubilized protein(s) and reincorporation of solubilized protein back into depleted thylakoids is minimized. The binding of solubilized proteins by asolectin and cholate is well known (1, 15, 24, 25, 29, 45).

Cation Effects on Reconstitution of V_{O_2}. The reconstitution of many membrane systems requires cations (28, 38, 46). The effect(s) of cations may be specific (*e.g.* activation) or nonspecific (electrostatic), depending on the system and the chemical properties of the ion (28, 38, 46). Preliminary experiments with the uncentrifuged, cholate-asolectin-extracted chloroplasts showed, however, that V_{O_2} of such chloroplasts remained unaffected following the addition and incubation with various concentrations of either mono- or divalent cations, thus indicating ineffectiveness of cations for inducing a direct reconstitution of V_{O_2}. Similarly, no reconstitution of V_{O_2} was obtained on dialysis of the extracted chloroplasts against ST buffer containing cations.

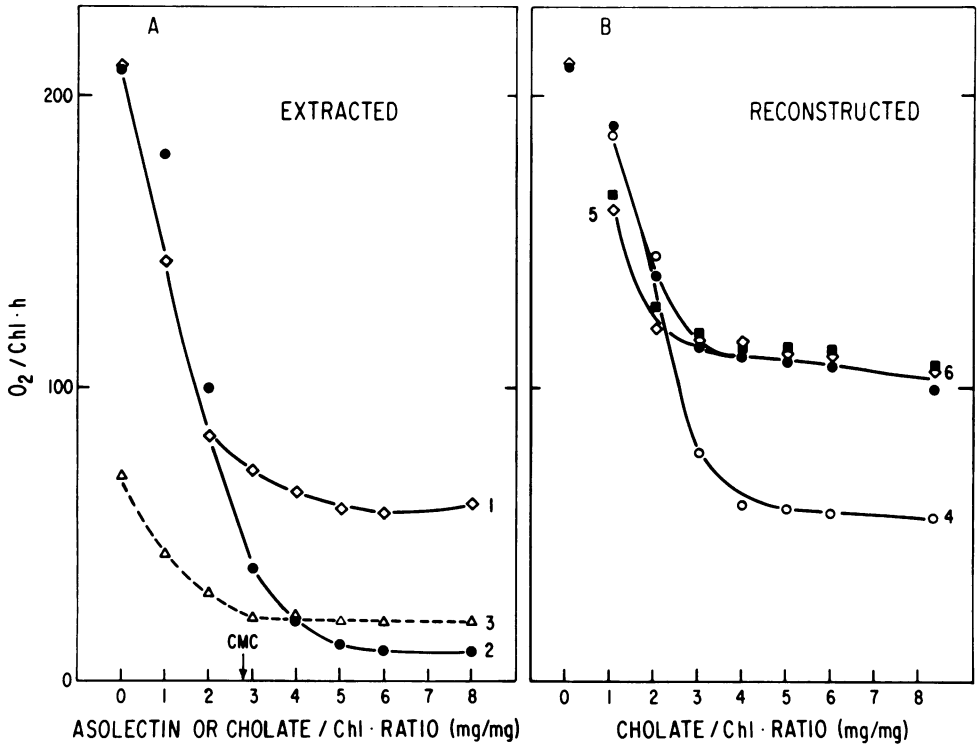

FIG. 6. Effects of asolectin on the cholate inactivation of Hill activity and its reconstitution by EG in the presence or absence of added chloroplast proteins. The extent of inactivation and reconstitution was determined over a range of cholate/Chl ratios. Chloroplasts (2 mg/ml) were extracted for 40 min, then assayed directly or following 40-min reconstitution in the presence of EG with and without added solubilized thylakoid proteins. A, V_{O_2} after extraction under the following conditions: curve 1, extraction at various cholate/Chl ratios in the absence of asolectin; curve 2, as for curve 1, except for inclusion of a fixed amount of asolectin (asolectin:Chl ratio of 4 on mg basis); and curve 3, extraction at a fixed amount of cholate (cholate:Chl of 4 on mg basis), but with varying amounts of asolectin. B, V_{O_2} following EG-induced reconstitution of the extracted chloroplasts of A: curve 5, reconstituted curve 1 chloroplasts with and without added solubilized thylakoid proteins, (solid and open symbols, respectively); curve 6, reconstituted curve 2 chloroplasts with added solubilized thylakoid proteins; and curve 4, reconstituted curve 2 chloroplasts without added solubilized thylakoid proteins. Protein was added where noted to yield a protein:Chl ratio of 1 on a mg basis.

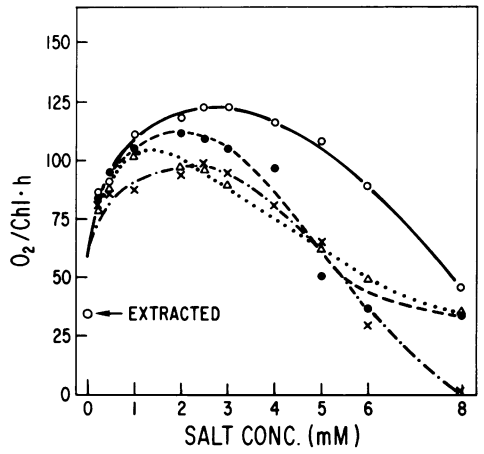

FIG. 7. Effects of salts of divalent cations on the EG-induced reconstitution of V_{O_2}, using cholate-asolectin-extracted chloroplasts in ST. Cations were added to the reconstitution medium at the time of EG addition. Control rate of V_{O_2} was 270 O_2/Chl·h (see "Materials and Methods"). Respective salts are: $CaCl_2$ (○——○); $MgCl_2$ (●——●); $MgSO_4$ (△···△); and $MnCl_2$ (X– · –X).

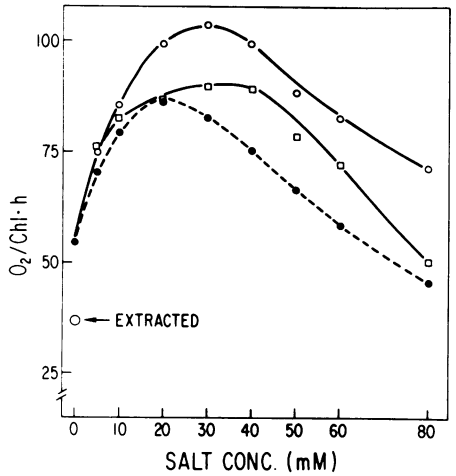

FIG. 8. Effects of salts of monovalent cations on the EG-induced reconstitution of V_{O_2}, using cholate-asolectin-extracted chloroplasts in ST. Cations were added to the reconstitution medium at the time of EG addition. Control rate of V_{O_2} was 240 O_2/Chl·h (see "Materials and Methods"). Respective salts are NaCl (○——○), KCl (□——□), and KNO_3 (●– –●).

In contrast, however, the addition of mono- or divalent cations to cholate-asolectin-extracted chloroplasts in ST buffer just prior to the addition of EG revealed marked enhancements of the reconstitution of V_{O_2}. Occasionally, with some lots of sucrose, prior decationization (Ag50-H^+) of the sucrose was required to observe maximum effects of the cations on reconstitution. Figures 7 and 8 show the effects of increasing concentrations of di- and monovalent salts, respectively, on the two-stage, EG-induced re-

constitution of V_{O_2}.

Figure 7 shows that reconstitution in the absence of added divalent cations yielded a 1.7-fold increase in V_{O_2}, but increasing divalent salt concentrations resulted in further increases of apparent reconstitution. Maximal effects for the divalent salts, with no apparent high order of specificity for either cation or anion, were obtained at ≤3 mM, with subsequent inhibition at higher concentrations of all the divalent salts. In this experiment, as much as 2.1-fold increase of apparent reconstitution was observed specifically from 3 mM $CaCl_2$, leading to an overall reconstitution of 3.5-fold and a V_{O_2} equivalent to ~52% of the control (unextracted) chloroplasts. According to previous arguments (Table I; Fig. 6), this reconstituted rate represents the maximal level of reconstitution that we could expect. We noted that such maximal levels of reconstitution were obtained without supplementation of thylakoid-solubilized proteins within the extraction milieu. We also noted that cation effects on reconstitution were not observed with chloroplasts subjected to extraction with cholate only, a result implicating specific cation-asolectin liposome interaction in the EG-induced reconstitution process.

The data of Figure 8 were obtained entirely similarly to those of Figure 7, except for use of monovalent salts. These data show that monovalent salts also enhanced reconstitution but that higher concentrations (~40 mM; the ST buffer containing 9.6 mM Na^+) were required, and about 20% less maximum reconstitution was obtained relative to results with Ca^{2+}.

These effects of cations relate to reconstitution of V_{O_2} *per se* and not to activation of PSII reaction centers (5) or to regulation of excitation energy (4), since rates of PSII-NH_2OH photooxidation of extracted or reconstituted thylakoids at quantum yield intensities were unaffected by concentrations of cations yielding maximal effects on reconstitution of V_{O_2} (data not shown).

We noted in the preceding experiments (Fig. 7) that maximum obtainable reconstitution was obtained in the presence of ~3 mM Ca^{2+} without the addition of supplemental solubilized thylakoid protein to uncentrifuged, liposome-extracted chloroplasts. Such results contrasted markedly with those of Figure 5, obtained in the absence of cation, showing a dependence of reconstitution on addition of supplemental protein. These contrasting results suggested that cations might increase the effectivity in reconstitution of the thylakoid-solubilized protein(s) in the extraction milieu, thereby eliminating the necessity of adding supplemental solubilized thylakoid protein to obtain maximum reconstitution. This supposition was supported indirectly by the observation that no EG-induced reconstitution of V_{O_2} was obtained by addition of cations without solubilized protein(s) to extracted thylakoid membranes purified by the density gradient ultracentrifugation.

In the experiments of Figures 9 and 10, we examined the effects of increasing concentrations of Ca^{2+} and Na^+, respectively, on reconstitution of V_{O_2} of cholate-asolectin-extracted, uncentrifuged chloroplasts in the presence and absence of supplemental solubilized thylakoid protein(s). Although the designated cation concentrations were included in the extraction as well as in the subsequent EG-induced reconstitution, the results obtained permit definitive conclusions regarding cation effects on reconstitution of V_{O_2}.

The data of Figure 9 show that, in the absence of Ca^{2+}, the addition of EG alone to the uncentrifuged, cholate-asolectin-extracted chloroplasts led to a significant increase of V_{O_2} but that supplemental, solubilized thylakoid protein yielded an additional increase of V_{O_2} to a level approaching the maximum expected level. Such results are entirely consistent with results in previous sections.

We note that the inclusion of Ca^{2+} (Fig. 9), but not of Na^+ (Fig. 10), in the extraction milieu led to slight inhibition of inactivation of V_{O_2} of extracted chloroplasts, thus obscuring the maximum magnitudes of ΔV_{O_2} observed on reconstitution (plus Ca^{2+} and without supplemental protein) of chloroplasts extracted in the absence of Ca^{2+} (Fig. 7). Nevertheless, the data clearly show that

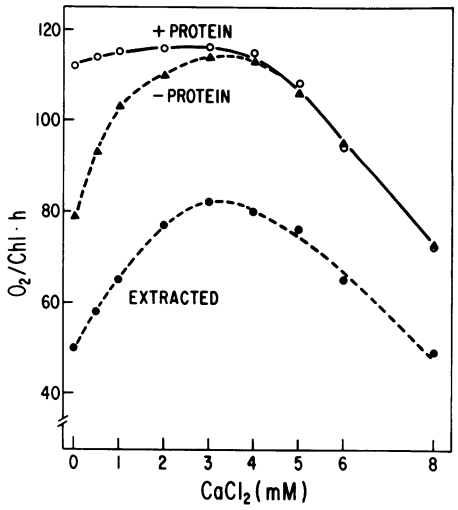

FIG. 9. Effects of $CaCl_2$ on the EG-induced reconstitution of V_{O_2} (in the presence and absence of supplemental thylakoid proteins [protein:Chl ratio, 1:1]), using cholate-asolectin-extracted chloroplasts in buffer containing $CaCl_2$. Control rate of V_{O_2} was 280 O_2/Chl·h (see "Materials and Methods").

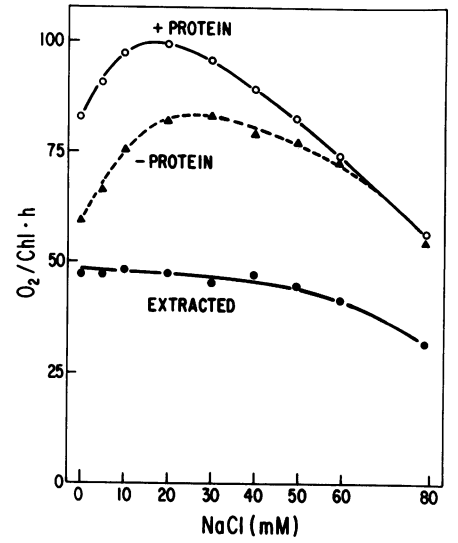

FIG. 10. Effects of NaCl on the EG-induced reconstitution of V_{O_2} (in the presence and absence of supplemental thylakoid proteins [protein:Chl ratio, 1:1]), using cholate-asolectin-extracted chloroplasts in buffer containing NaCl. Control rate of V_{O_2} was 260 O_2/Chl·h (see "Materials and Methods").

the ΔV_{O_2} obtained in response to supplemental, solubilized thylakoid protein (plus protein, Fig. 9) diminished to zero as Ca^{2+} concentrations approach 3 to 4 mM. Similar conclusions, except for differences in cation concentration requirements, can be drawn from Figure 10, which shows the effect of Na^+ on reconstitution of V_{O_2} in the absence and presence of supplemental, solubilized thylakoid protein.

We interpret the results of Figures 7 through 10 to indicate that cations promote reconstitution by increasing the availability of solubilized thylakoid protein within the extraction milieu to the depleted thylakoids. Such increased availability could be a result of either cation-induced release of bound or sequestered, solubilized proteins from the liposomes or a cation-induced fusion of liposomes containing solubilized proteins with the depleted thylakoids (15). Such cation effects (Fig. 7–10) clearly are nonspecific and, thus, probably are of electrostatic character (46).

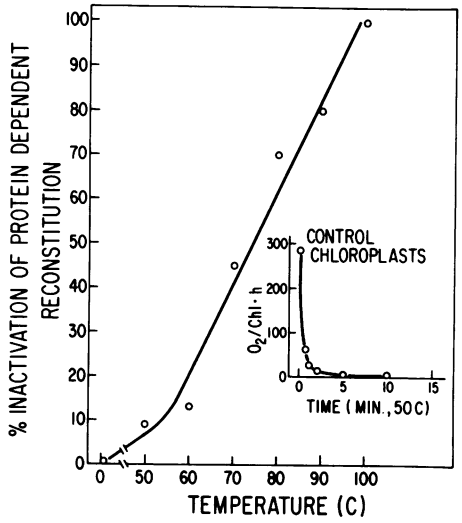

FIG. 11. Comparisons of temperature sensitivity of protein fraction effecting reconstitution with inactivation of V_{O_2} of control (unextracted) chloroplasts. Control chloroplasts (1 mg/ml equivalent to ~8 mg total protein/ml) were heated in ST buffer at 50°C for the times indicated (inset). The protein fraction (8 mg/ml) was obtained as described in "Materials and Methods" and was added (protein:Chl ratio, 1:1) just prior to reconstitution following a 5-min treatment at temperatures indicated on abscissa. V_{O_2} of cholate-asolectin-extracted and reconstituted thylakoids in the absence and presence of supplemental protein(s) were 69, 80, and 120 O_2/Chl·h, respectively.

Table V. *Comparison of the Effectivity of Protein Fractions in the Reconstitution of V_{O_2} of Cholate-Asolectin-Extracted Chloroplasts*

V_{O_2} of control chloroplasts was 315 O_2/Chl·h. For other details, see "Materials and Methods."

Addition	V_{O_2}	Increase of V_{O_2} from Added Protein[a]
		O_2/Chl·h
ST buffer	75	
EG	108	
Spector-Winget (44) extracted protein fractions (300 μg), then EG		
$(NH_4)_2SO_4$ pellet, 0.84 M	125	17 (0.30)[b]
$(NH_4)_2SO_4$ pellet, 1.2 M	122	14 (0.25)
$(NH_4)_2SO_4$ pellet, 2.0 M	135	27 (0.48)
Cholate-asolectin-extracted protein (2.0 M $[NH_4]_2SO_4$ pellet [300 μg]) then EG	164	56 (1.0)

[a] EG plus protein minus EG alone.

[b] Values in parentheses represent relative effectiveness of added protein in reconstitution of V_{O_2}.

General Properties of the Solubilized Thylakoid Protein(s) Effective in Reconstitution of V_{O_2} of Cholate-Asolectin-Extracted Chloroplasts. The following evidence indicates that the solubilized thylakoid protein(s) effective in the reconstitution of V_{O_2} described here is not identifiable with the Mn-containing, O_2-evolving enzyme (37): solubilization of the proteins is not accompanied by solubilization of functional Mn (Table II); and the protein fraction effecting reconstitution (Table II) does not contain Mn and, moreover, is insensitive to NH_2OH (41), a reagent effecting release of the Mn associated with V_{O_2} *per se* (9, 26, 37, 42). The data of Figure 11 reinforce this conclusion, while the data of Table V indicate that we do not deal with either the 65-kd (44) or the 232-kd (58-kd monomer) (35, 36) proteins that effect reconstitution of V_{O_2} in the system described by Spector and Winget (44) and/or that by Nakatani and Barber (35, 36), respectively.

Figure 11 compares the temperature sensitivity (5-min treatment at temperature designated on abscissa) of the protein fraction effecting reconstitution with the inactivation of V_{O_2} of control (unextracted) chloroplasts (Fig. 11, inset) by 50°C treatment. Such 50°C treatment rather specifically inactivates the S-state complex with release of functional Mn (8, 12, 37, 49, 51). The data show that 5 min at 50°C completely abolished V_{O_2} of control chloroplasts but caused only ~10% loss of the effectiveness of supplemental protein for reconstituting V_{O_2} of extracted chloroplasts. Increasing temperatures, however, yielded further denaturation of the protein(s), such that no effect of the supplemental protein on reconstitution was obtained after 100°C treatment. The results further indicate that we do not deal with the S-state Mn 'enzyme' but rather with a protein(s) of rather high thermal stability.

As previously indicated, two different proteins have been reported to yield reconstitution of V_{O_2} of chloroplasts subjected to the cholate extraction procedures of Spector and Winget (44): a 65-kd protein reported to contain Mn (44) (see, however, Refs. 35, 36, and 41); and an Fe-heme 232-kd protein (58-kd monomer) (35, 36). Both of these proteins can be partially purified by precipitation with $(NH_4)_2SO_4$ in repeated steps, using first 1.2 M, then, after solution of precipitated proteins, 0.84 M $(NH_4)_2SO_4$ precipitation (35, 36, 44). We compared the effectiveness of such extracted and precipitated proteins with a 2.0 M $(NH_4)_2SO_4$ precipitate of solubilized proteins from the cholate-asolectin-extracted chloroplasts for the reconstitution of V_{O_2} in cholate-asolectin-extracted, uncentrifuged chloroplasts in ST buffer only. In this protocol, the total amount of protein-dependent reconstitution is the sum of the effects of thylakoid protein solubilized plus effects from any solubilized thylakoid added just prior to reconstitution *per se*.

Addition of EG alone to cholate-asolectin-extracted chloroplasts increased V_{O_2} slightly (75 to 108 O_2/Chl·h), and the addition of a 2.0 M $(NH_4)_2SO_4$ precipitated protein fraction from cholate-asolectin-extracted proteins increased total reconstitution 2.2-fold to an extent equivalent to 52% of control (unextracted) chloroplasts (Table V). Meaningful comparisons of these data with the other protein fractions shown in Table V can be made, if we compare increase of V_{O_2} from equivalent amounts of protein fractions added just prior to reconstitution.

The data in Table V indicate that solubilization of thylakoid proteins and precipitation by procedures of Spector and Winget (44) or Nakatani and Barber (35, 36) yield proteins (0.84 and 1.2 M $[NH_4]_2SO_4$ pellets) which are only 25 to 30% as effective in reconstitution of V_{O_2} of our extracted chloroplasts as are the protein fraction from cholate-asolectin-extracted chloroplasts. We conclude that the protein(s) effecting the reconstitution of V_{O_2} reported here is dissimilar from the 65- or the 232-kd (58-monomer) proteins. This conclusion is supported by the data of Figure 2: the 1.2 M and 0.84 M $(NH_4)_2SO_4$-precipitated protein fractions used in Table V contained 65- and 58-kd proteins (in the latter case, however, the data do not permit discrimination between the Fe-heme subunit and the α-or β-subunit of CF_1); and the polypeptide profiles of solubilized proteins obtained by the two extraction procedures show few similarities.

DISCUSSION

The results presented here indicate that extraction of chloroplasts with cholate or cholate-asolectin liposomes at specified conditions results in a rapid and rather selective inactivation of V_{O_2}, which then can be reversed partially or reconstituted with proteins extracted from thylakoids. A similar general inactivation and reconstitution of V_{O_2} has been reported previously by Spector and Winget (44), using entirely different procedures for both extraction and reconstitution and yielding conclusions different than those obtained here.

The extraction procedures used here (liposomes in the absence

of Mg^{2+}) do not result in solubilization of the functional Mn of the S-state complex concomitant with loss of V_{O_2} and do not grossly affect PSI donor activity or, apparently, the e transport between PSII and PSI, but they do yield partial (~50%) inactivation of PSII donor activities. In contrast, the extraction procedures employed by Spector and Winget (44) resulted in a presumed solubilization of functional Mn with loss of V_{O_2} and complete inactivation of PSII as well as PSI donor activities which, however, were reactivated by simple incorporation of extracted thylakoids into artificial liposomes in the absence of supplemental thylakoid-derived protein. In our hands (41), however, procedures of Ref. 44 yielded no significant solubilization of functional Mn. Likewise, rather high concentrations of deoxycholate also do not solubilize functional Mn, but they do yield loss of V_{O_2} but not of PSII donor activities (2).

We interpret the extraction-reconstitution results presented here to indicate a reversible decoupling of the Mn-containing, S-state complex from its connection with native donor(s) to P680 that is not inactivated by the extraction procedure. This interpretation is based on: (a) lack of solubilization of functional Mn and minimum solubilization of the reported Tris-sensitive, Mn-containing, 65-kd protein (44) or the Fe-heme, 58-kd (232-kd oligomer) protein, implicated by Nakatani and Barber (35, 36) to function in V_{O_2}; (b) a partial reconstitution of V_{O_2} by a protein fraction depleted of the 65- and 58-kd polypeptides and having greater specific activity than protein fractions enriched in these polypeptides reportedly containing Mn and Fe, respectively; and (c) the reconstitution of V_{O_2} without reconstitution of the ~50% loss of PSII donor activity occurring in our extractions.

In contrast, the extraction-reconstitution studies of Spector and Winget (44), using higher cholate-to-Chl ratios than are used here, imply a reversible decoupling of the S-state complex itself from the oxidizing side of PSII with reconstitution of V_{O_2} obtained with only a highly purified Mn, 65-kd protein. On the other hand, a minimum reconstitution of V_{O_2} is obtained with an Mn-free, Fe-heme, 58-kd protein (35, 36), using the extraction-reconstitution procedures of Ref. 44. Unfortunately, we have been unable, so far, to reproduce these results, thus excluding direct comparisons and/or 'hybridization' experiments, using these two procedures with the aim toward identification of the proteins associated with the S-state complex, secondary donors to P680, and intrinsic proteins of the PSII membrane complex.

The data presented here do not exclude a role of a 34-kd polypeptide in our reconstitution of V_{O_2}. This polypeptide has been implicated to be involved in the binding of functional Mn from the analyses of specific non-O_2-evolving mutants of *Scenedesmus* (32, 33). Indeed, the solubilized thylakoid protein fraction yielding the reconstitution of V_{O_2} reported here does contain polypeptides in the 34-kd region (as well as those of PSII origin [10]). Moreover, the solubilization of the 34-kd polypeptide(s) by liposomes is diminished in extractions (plus EG), which severely limit loss of V_{O_2} (Table I; Fig. 2). We note, however, the solubilization of polypeptides in the 34-kd region, by either the extraction procedure used here or those of Ref. 44, but without cosolubilization of functional Mn (Fig. 2; Table II) (40). Assuming coidentity (not specifically demonstrated) of the 34-kd bands observed by us and Bishop's group (32, 33), we question the assignment of the 34-kd protein as a Mn-protein.

The extraction-reconstitution procedures employed here are somewhat unusual on two accounts: the inclusion of artificial liposomes in the extractions of chloroplasts with cholate, as well as in the reconstitution; and the use of mono- or dihydroxy-alcohols in reconstitution *per se*. The data of Figure 6 (A and B) suggest that artificial liposomes are not absolutely essential for either abolishment of V_{O_2} or the subsequent EG-induced reconstitution of V_{O_2}. These data indicate that the liposomes increase the observed extent of killing of V_{O_2} without modifying the maximum observable extent of reconstitution. It is not entirely clear whether this increased killing of V_{O_2} by liposomes is due solely to a sequestering of solubilized proteins on or within the liposomes or to observed qualitative differences in proteins solubilized. We tend to favor the first alternative, based on the data of Figures 7 through 10, showing that cations can maximize the EG-induced reconstitution dependent on solubilized proteins within the chloroplast-liposome extractions, thereby eliminating the requirement for supplemental, solubilized protein. This interpretation envisions either a cation-induced desorption of solubilized protein from the liposomes or an enhanced fusion of the liposome-containing protein with depleted thylakoids.

To our knowledge, the use of solvents like EG for reconstitution of V_{O_2} is the first reported reconstitution of a membrane function by organic solvents. Such reconstitution of V_{O_2} avoids the use of commonly used sonication procedures for reconstitution of membrane function (35, 44). It is commonly known that sonication results in inactivation of O_2 evolution. The use of EG in reconstitution of V_{O_2} was predicated on the fact that this solvent is innocuous to most reactions of chloroplasts (16) but causes degeneration of cholate micelles (31). Such solvent-induced degeneration of micelles is analogous to that obtained by dialysis or dilution, two commonly used techniques for reconstitution of membrane systems. However, because of its high polarizability, EG also is believed to increase, relative to H_2O, hydrogen bonding and electrostatic interactions while decreasing solvophobic interactions within macromolecules (17, 18, 22, 23). Which of these effects of EG is critical for the reconstitution of V_{O_2} is unknown.

The totality of the results presented here suggest minimally two sites of inactivation on the oxidizing side of PSII exclusive of the S-state complex. These results indicate that only one of these inactivated sites is reconstitutable, using the described methodologies. In the absence of kinetic data on Z_1 and Z_2 (6, 13, 27, 39, 49) for the extracted and reconstituted thylakoids and chemical characterization of the protein(s) yielding reconstitution, we cannot as yet assign a definitive function (structural *versus* electron transfer component) to the solubilized protein(s) required for the reconstitution of V_{O_2}.

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