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Purification of a manganese-containing protein involved in photosynthetic oxygen evolution and its use in reconstituting an active membrane

(photosystem II/spinach chloroplast/water oxidation)

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ABSTRACT **Extraction of thylakoid membranes with cholate in the presence of ammonium sulfate inactivated oxygen evolution and liberated a manganese-containing protein. This protein could be combined with preformed liposomes containing the depleted thylakoid membranes to restore 85% of the original oxygen-evolution activity. The protein did not affect the primary photochemical events of photosystem I or photosystem II, and it was required only for electron transport in which water was the electron donor. The protein has been purified to homogeneity and has an apparent molecular weight of 65,000 (polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate). Atomic absorption revealed two atoms of manganese bound to each 65,000-dalton protein molecule. Treatment with alkaline Tris removed the bound manganese and rendered the protein incapable of restoring oxygen evolution; however, Tris treatment of the depleted membranes before reconstitution had no effect. Thus, this manganese protein is probably the site of Tris action in isolated chloroplasts and is at least part of the water-oxidation enzyme system.**

The chemical events leading to the photosynthetic evolution of oxygen are still largely a matter of speculation. Many aspects of the kinetics of oxygen evolution have been elucidated. A model involving the storage of oxidizing equivalents on an unknown enzyme (the "S-states") has been proposed and seems to have gained general acceptance (1). The photoactivation of an uncharacterized manganese-containing protein may be essential for oxygen evolution (2, 3). In addition, the specific requirement for manganese in the oxygen-evolving reaction has been demonstrated by inducing deficiency during growth or by depleting manganese by extraction, in both algae and higher plants (4, 5).

A subchloroplast system in which depletion and restoration of oxygen evolution can be accomplished by the extraction and replacement of individual thylakoid components would be helpful in furthering our understanding of photosynthetic oxygen evolution. We present here a method for extracting a manganese-containing protein and using it to reconstitute an active system; our results provide evidence that this protein is required for photosynthetic oxygen evolution.

METHODS AND MATERIALS

Chloroplasts were isolated from fresh market spinach and extracted with cholate (6). Briefly, specific chloroplast lipids and proteins were solubilized by using 0.05 M sodium cholate and 0.4 M ammonium sulfate at pH 8.0 with stirring for 15 min on ice. The suspension was then centrifuged at 144,000 × *g* for 90 min, and the supernatant was collected; the pellet was set aside. Saturated ammonium sulfate (pH 8.0) was then added to the supernatant to increase the concentration to 1.2 M. After incubation for 20 min on ice the precipitate was collected by centrifugation (10,000 × *g*, 10 min). This pellet was dissolved in about 3 ml of 0.2 M sucrose/0.02 M *N*-[tris(hydroxymethyl)methyl]glycine (Tricine)–NaOH (pH 8.0)/3 mM $MgCl_2$, then adjusted to 0.84 M with saturated ammonium sulfate (pH 8.0). After centrifugation, the pellet was again dissolved in buffered sucrose. Further purification was accomplished by chromatography on a Sephadex G-200 (medium) column (2.0 × 50 cm) and rechromatography of the fraction active in restoring oxygen evolution on a Sephadex G-100 (medium) column of the same size. Except for the initial extraction with cholate, no detergent was used in the purification steps.

The preparation of chlorophyll-containing liposomes was accomplished by a 15-s sonication of about 0.5 ml of the depleted thylakoid membranes (the 144,000 × *g* pellet after cholate extraction, see above) with 0.5 ml of preformed liposomes (sonicated soybean phospholipids, asolectin, 20 mg/ml, see ref. 7) to give a final chlorophyll concentration of about 5 mg/ml. These chlorophyll-containing liposomes were passed through a Bio-Gel A-1.5 m column (2.0 × 40 cm). The liposomes eluted as a single band. The chlorophyll concentration in the suspension was determined at this point. Electron microscopy showed a homogeneous population of 25- to 50-nm vesicles, which we refer to as depleted photosomes (electron micrographs not shown). Fifty-microliter fractions containing the manganese protein at different stages of purification were sonicated with 0.2 ml of the depleted photosomes for 6 s, incubated for 30 min on ice, then assayed for oxygen evolution by using a YSI model 53 oxygen monitor with Teflon-covered Clark-type oxygen electrode. Although the values given are for initial rates, oxygen evolution usually continued at close to the rate reported for several minutes or until the electron acceptor became fully reduced. Illumination with heat-filtered white light at saturating intensity was provided by a 750-W projector focused on the electrode chamber.

Manganese was determined by atomic absorption analysis of the purified and dialyzed protein. The protein was dissolved in 2 mM potassium phosphate buffer (pH 7.5) and dialyzed against 1000 vol of distilled water for 10 hr at 4°C. The protein was lyophilized in 25-μg amounts for analysis.

Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed by the method of Chua and Bennoun (8).

Protein was determined by the method of Lowry *et al.* (9). Chlorophyll was determined as described by Arnon (10).

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Abbreviations: DPC, diphenylcarbohydrazide; Cl_2indophenol, 2,6-dichloroindophenol; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

For the removal of manganese, photosomes were first treated with 0.8 M Tris·HCl (pH 8.0) for 15 min under bright white light (11), then pelleted by centrifugation at 144,000 × *g* for 30 min, and the pellet was resuspended in a Tris-free buffer. A similar Tris treatment was used to treat the purified protein, except that the protein (2 mg/ml) was precipitated from the Tris solution by 2 M ammonium sulfate (pH 8.0).

Cholic acid was obtained from Sigma and was twice recrystallized from 70% aqueous ethanol after treatment with Norit A charcoal. Asolectin was obtained from Associated Concentrates, Woodside, NY, and was freed of acetone-soluble and ether-insoluble materials. Ultrapure ammonium sulfate and enzyme grade sucrose were obtained from Becton-Dickinson Immunodiagnostics, Orangeburg, NY. All other chemicals were reagent grade.

RESULTS

Depleted photosomes catalyzed high rates of photosystem II activity measured as electron transport from diphenylcarbohydrazide (DPC) to 2,6-dichloroindophenol (Cl_2indophenol) and photosystem I activity measured as electron transport from reduced Cl_2indophenol to methyl viologen. However, there was no measureable oxygen evolution in the presence of Cl_2indophenol (i.e., there was no Hill reaction). This indicated a specific loss of the activity of a component on the oxidizing side of photosystem II, before the site of DPC oxidation. However, after reconstitution with the 65,000-dalton, manganese-protein, the photosomes catalyzed high rates (typically over 800 μeq/hr·mg chlorophyll) of methylamine-uncoupled oxygen evolution. The results of one such reconstitution experiment are given in Table 1. The ability of the 65,000-dalton protein to restore Hill activity was lost when it was treated with alkaline Tris (pH 8.0), but a similar treatment of photosomes prior to reconstitution with the protein had no effect.

Table 1. Effect of purified 65,000-dalton protein on partial electron transport reactions of depleted photosomes

Components	Reaction rate,* μeq/hr·mg chlorophyll: Water to Cl_2indophenol	DPC to Cl_2indophenol	Reduced Cl_2indophenol to methyl viologen
144,000 × *g* precipitate	0	0	0
Depleted photosomes	0	296	1198
Depleted photosomes plus 65,000-dalton protein	360 (892)	23	1087
Depleted photosomes plus Tris-treated 65,000-dalton protein	42	284	1146
Tris-treated photosomes plus untreated 65,000-dalton protein	356	27	1043
Isolated chloroplasts	392 (996)	284	1264

All reaction mixtures contained 0.2 M sucrose, 0.04 M Tricine–NaOH (pH 8), 6 mM $MgCl_2$, and 60 μg of chlorophyll in a total volume of 2 ml. The reaction water to Cl_2indophenol was measured with an oxygen electrode; the mixture contained 0.4 mM Cl_2indophenol. The mixture for the reaction from DPC to Cl_2indophenol contained 0.5 mM DPC and 0.1 mM Cl_2indophenol, and the reaction was measured spectrophotometrically by following Cl_2indophenol reduction at 590 nm. The reaction reduced Cl_2indophenol to methyl viologen was measured by following oxygen uptake in a Mehler-type reaction with an oxygen electrode. The latter reaction took place in the presence of 5 mM ascorbic acid, 0.4 mM Cl_2indophenol, 50 μM methyl viologen, and 1 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

* Rates given in parentheses were measured in the presence of uncoupler (20 mM methylamine).

Table 2. Purification of the 65,000-dalton manganese-containing protein

Material	Total protein, mg	Total units, μmol O_2/hr	Specific activity, μmol/hr·mg
Chloroplasts	1766	37,600	21.3
1.2 M $(NH_4)_2SO_4$ fraction	874	33,200	38.1
0.84 M $(NH_4)_2SO_4$ fraction	226	28,600	126.5
Pooled Sephadex G-200 fraction	52.5	15,200	289
Pooled Sephadex G-100 fraction	6.2	4,850	782

The steps in the purification of the oxygen-evolution factor are summarized in Table 2. The protein nature of the factor was confirmed by its sensitivity to trypsin digestion. About 6 mg of the purified protein was obtained from spinach chloroplasts containing 392 mg of chlorophyll. This protein represented about 13% recovery of the activity (oxygen evolution) of the original chloroplasts but showed a more than 37-fold increase in specific activity. The purified protein gave a single band on polyacrylamide gel electrophoresis in the presence of Na-$DodSO_4$, indicating an apparent molecular weight of 65,000 (Fig. 1).

The presence of manganese in the protein was confirmed by the use of spinach grown with ^{54}Mn supplementation (data not shown), and the amount was determined by quantitative manganese analysis using atomic absorption spectroscopy

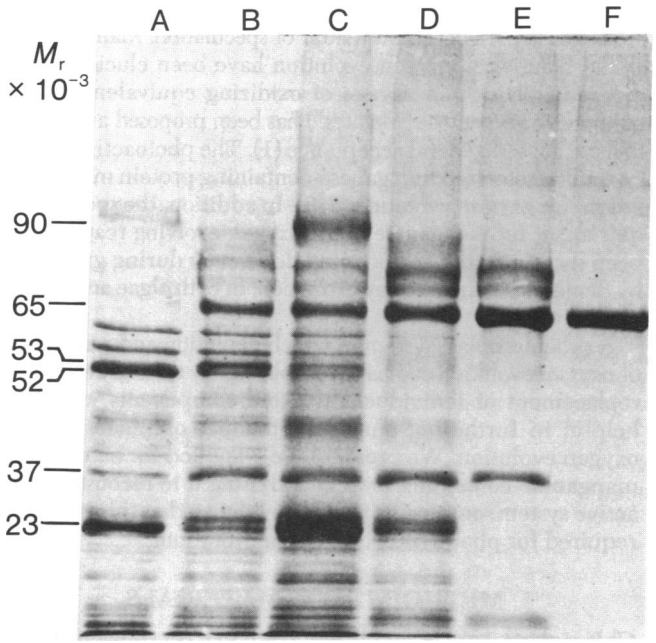

FIG. 1. Coomassie-blue stained proteins separated by Na-$DodSO_4$/polyacrylamide gel electrophoresis at different stages of purification of the manganese-containing protein from spinach chloroplasts: lane A, isolated chloroplasts; lane B, supernatant after cholate extraction of chloroplasts; lane C, pellet from 1.2 M ammonium sulfate precipitation of B; lane D, pellet after reprecipitation of dissolved pellet from C with 0.84 M ammonium sulfate; lane E, active fraction collected by passing redissolved pellet (from D) over Sephadex G-200 column; lane F, active fraction collected after passing material from E over Sephadex G-100 column. See text for details of fractionation procedure.

Table 3. Atomic absorption analysis of manganese content of 65,000-dalton protein

Sample	Protein, μg	nmol protein per ml in 0.01 M HNO_3	ng Mn per ml	mol Mn per mol protein
1	25	0.192	28	2.65
2	25	0.192	17	1.61

Samples were dialyzed and lyophilized before analysis.

(Table 3). Manganese analysis indicated that there were two manganese atoms bound to each 65,000-dalton protein molecule. Moreover, Tris treatment released radioactive manganese (^{54}Mn), leaving a manganese-free apoprotein (data presented elsewhere).

DISCUSSION

Successful attempts to restore oxygen evolution from inactivated chloroplasts previously have been limited to the reincorporation of manganese into binding sites on the thylakoid membrane (12). Several soluble factors have been reported to stimulate the Hill reaction in whole chloroplasts (13, 14); however, only manganese, calcium, and chloride have been definitely shown to be involved in oxygen evolution (5). An 11,000-dalton polypeptide was purified by preparative polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ by Schmidt and coworkers, and antisera prepared against this polypeptide inhibited oxygen evolution but not the oxidation of secondary donors in sonicated chloroplasts (15). In general, detergent treatments destroy the oxygen evolution centers. Cholate, too, disrupts oxygen evolution, but does not solubilize the chlorophylls, cytochromes, or many other of the integral proteins and lipids of the thylakoid membrane (16). Thus, the pellet at 144,000 × *g* represents membranes depleted of specific proteins, such as the ATPase complex (6) and the manganese protein described here.

Insertion into the membrane vesicle is essential for oxygen evolution activity, presumably due to a specific native conformation that is imparted by the lipid bilayer environment. However, our results should not be used to indicate that a vesicle is necessary for activity, even though a similar proposal has been made previously (17).

Yamashita and Butler (18) showed that treatment with alkaline Tris inactivates the oxygen-evolving step. Later, the loss of a specific fraction of the membrane-bound manganese was correlated with the Tris inactivation phenomenon (19). Electron spin resonance spectroscopy confirmed that Mn^{2+} was released from a membrane-bound state during Tris treatment (20).

Although other proteins involved in photosynthetic electron transport probably contain manganese as a ligand, and especially those on the oxidizing side of photosystem II, the Tris sensitivity of this particular protein suggests that it is the site of Tris inhibition observed in chloroplasts, and thus is an integral component of the oxygen-evolving complex.

The reconstituted vesicles we have termed photosomes showed stimulation of the Hill reaction in the presence of methylamine and 3-*tert*-butyl-5,2′-dichloro-4′-nitrosalicylanilide (S-13). This indication of the formation of a proton gradient may make this system useful as well for the study of reconstitutions of electron transport-linked photophosphorylation reaction systems.

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