Use of Chemical Modification To Study the Relationship between Activity and Net Protein Charge of the Photosystem I Core Complex†

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ABSTRACT: The net charge on the photosystem I core complex of Shiozawa et al. (Shiozawa, J. A., Alberte, R. S., & Thornber, J. P. (1974) *Arch. Biochem. Biophys. 165*, 388–397) has been altered by using a water-soluble carbodiimide to form an amide bond between protein carboxyl groups and an amino group of ethylenediamine. This process replaces negatively charged carboxyl groups with positively charged free amino groups. Six hundred moles of ethylenediamine was incorporated per mole of P700 reaction center. The modification of the complex shifted the isoelectric pH of photosystem I from 5.0 to 9.5 without affecting the total amount of P700. The modified complex exhibited an increase in energy transfer from light-harvesting chlorophyll *a* molecules to the reaction center. This phenomenon has been previously observed upon addition of Mg^{2+} ions to the complex (Gross, E. L., & Grenier, J. (1978) *Arch. Biochem. Biophys. 187*, 387–398). The modification replaces the divalent cation requirement for electron donation to P700 by plastocyanin and lowered the K_m for plastocyanin binding to 2.0 μM, compared to 32 μM for control photosystem I in the presence of Mg^{2+}. In addition, the modification lowered the K_m for the negatively charged electron donors dichlorophenolindophenol and ascorbate. These results suggest that changing the charge on the photosystem I complex from negative to positive stimulates both light utilization and electron transfer from electron donors to P700. We suggest that cation regulation of photosystem I activity occurs by a process in which cations alter the charge of the local environment around the complex.

It is well established that cations regulate grana stacking (Izawa & Good, 1966; Anderson & Vernon, 1967; Gross & Prasher, 1974) and energy distribution (Murata, 1971; Jennings & Forti, 1974; Marsho & Kok, 1974; Barber & Mills, 1976; Gross et al., 1976) in isolated chloroplasts. This cation regulation is complicated in that monovalent and divalent cations have antagonistic effects which are dependent on salt concentration and chloroplast isolation procedure (Gross & Hess, 1973; Vandermeulen & Govindjee, 1974). Cation regulation may occur at two levels. The first level requires cooperation of both photosystems and their interaction with the light-harvesting chlorophyll *a*/*b* protein (LHC).[1] The second level of cation regulation occurs within each individual photosystem.

Our laboratory has been studying cation regulation at the second level by examining subchloroplast particles containing only one photosystem. Davis et al. (1977) found that divalent cations decreased quantum yield for electron transport in TSF-II particles which contain the reaction center of photosystem II in association with a large amount of LHC. Removal of LHC produced a photosystem II core complex (TSF-IIa) in which cations increased quantum yields for electron transport. LHC is therefore the site of cation inhibition of energy transfer in TSF-II.

Cations also regulate energy transfer within photosystem I (PSI). Addition of cations to PSI subchloroplast particles resulted in a decrease in chlorophyll *a* fluorescence accompanied by increased quantum yield for PSI electron transport (Prochaska & Gross, 1977). Gross & Grenier (1978) found that Mg^{2+} ions caused a 12% increase in the α-helical content of PSI. This information suggests that cations promote a structural change in PSI particles which results in an increase in the amount of excitation energy that reaches the reaction center. Cations have also been shown to regulate the interaction of PSI particles with artificial electron donors (Gross, 1979) and the natural electron donor plastocyanin (Davis et al., 1980).

The question arises as to how cations regulate activities within each individual photosystem. Both the chloroplast membrane (Nakatani & Barber, 1980) and isolated PSI particles (Satoh & Butler, 1978; Siefermann-Harms & Ninnemann, 1979) have a net negative charge at neutral pH values. We postulate that cations act by changing the net charge on the complex. If this is true, then changing the net charge on PSI by using chemical modification techniques should mimic the action of cations. So that this hypothesis could be tested, the PSI complex was reacted with a water-soluble carbodiimide in the presence of ethylenediamine (see eq 1) (Means & Feeney, 1971). In this scheme, a negatively

$$\text{PSI-COO}^- + :\text{NH}_2(\text{CH}_2)_2\text{NH}_3^+ \xrightarrow[\text{pH 6.0}]{\text{carbodiimide}} \text{PSI-CONH}(\text{CH}_2)_2\text{NH}_3^+ \quad (1)$$

charged carboxyl group is replaced by a positively charged amino group.

Experimental Procedures

Preparation of PSI and Plastocyanin. PSI was isolated from spinach according to the method of Shiozawa et al. (1974) except that the protein was eluted from the hydroxylapatite column with 300 mM sodium phosphate buffer, pH 7.0, containing 0.05% Triton X-100. The PSI preparation was dialyzed by one of two procedures, depending on the intended use. The first procedure involved diluting the PSI 1:1 with double-distilled deionized water followed by dialysis against

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[1] Abbreviations used: PSI, photosystem I particles; EDA-PSI, photosystem I particles modified with ethylenediamine; chl, chlorophyll; LHC, light-harvesting chlorophyll *a*/*b* protein; P700, reaction center chlorophyll of photosystem I which absorbs maximally at 700 nm; $P700^+$, oxidized P700; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; DPCN, diphenylcarbazone; DCIP, 2,6-dichlorophenolindophenol; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

0006-2960/81/0420-2961$01.25/0

several changes of distilled water until the PSI precipitated. The precipitated PSI was collected by centrifugation at 30000*g* and resuspended in distilled water by using a hand homogenizer. The second procedure involved dialysis of the PSI against three changes of 0.05% Triton X-100.

Chlorophyll concentrations were determined by the method of Arnon (1949). The final PSI preparations had chl/P700 ratios of 100–150 and chl *a*/chl *b* ratios of 6–8.

Plastocyanin was isolated according to the method of Davis & San Pietro (1979). The purified plastocyanin had a final A_{275}/A_{597ox} ratio of 1.2–1.6 and eluted as a single peak by both Sephadex G-75 gel filtration and DEAE-cellulose ion-exchange chromatography using linear NaCl gradients. Plastocyanin concentrations were measured according to Davis & San Pietro (1979) using an extinction coefficient of 4.9 mM^{-1} cm^{-1} at 597 nm.

Chemical Modification of PSI. PSI was modified in the presence or absence of Triton X-100. Isolated PSI dialyzed against 0.05% Triton X-100 was used for reactions in which the protein was modified in the presence of this detergent. PSI (1–2 mg of chl) was added to a solution of ethylenediamine dihydrochloride which had been previously adjusted to pH 6.0 with NaOH. This mixture was adjusted to pH 6.0 ± 0.05. EDC was added to this reaction mixture in the form of a concentrated stock solution prepared immediately before use. The final concentrations of the components in the reaction were PSI containing 20 μg of chl/mL, 0.2 M ethylenediamine, and 0.1 M EDC. Triton X-100 was added to a final concentration of 0.05% for modification performed in the presence of this detergent. The reaction mixture was stirred at room temperature for 1 h at pH 6.0 ± 0.1. The reaction was stopped by diluting the reaction mixture 1:1 with 0.5 M Tris–succinic acid buffer, pH 6.0.

The modified PSI was treated in one of two ways, depending on the Triton X-100 content. When the reaction mixtures contained no Triton X-100, the PSI was collected by centrifugation at 30000*g* at 4 °C followed by two washings with distilled water. The washed PSI pellet was homogenized in distilled water and stored at 4 °C. For reaction mixtures containing Triton X-100, the modified PSI was dialyzed at 4 °C against several changes of distilled water until the protein began to precipitate. The modified PSI was collected by centrifugation at 30000*g*, resuspended in distilled water, and stored at 4 °C.

For the labeling experiments, 5 μCi of [^{14}C]ethylenediamine was added to the reaction mixture containing unlabeled ethylenediamine before the addition of EDC. Radioactive ethylenediamine was also added to control reaction mixtures in order to follow the removal of free ethylenediamine during later dialysis. At some point during the 1-h reaction, aliquots of the control and modified reaction mixtures were removed and used to determine the specific activity (disintegrations per minute per micromole of ethylenediamine) of the mixture. After the modification procedure, PSI (50–100 μg of chl) and 0.6 mL of 30% H_2O_2 were combined in liquid scintillation vials in a total aqueous volume of 1.0 mL. The vials were capped and heated in an oven at 65 °C for 1.5 h to oxidize the chlorophyll. After the vials were cooled to room temperature, 10 mL of scintillation cocktail (Patterson & Green, 1965) was added to each vial. After mixing, the vials were placed in a Beckman LS-230 liquid scintillation counter, and the amount of incorporated ethylenediamine was determined.

Diphenylcarbazone (DPCN) Disproportionation. The assay mixture contained control or modified PSI (10 μg of chl/mL), 10 mM Tris-HCl, pH 8.2, 0.15 mM DPCN, and 0.05% Triton X-100. DPCN disproportionation was monitored at 485 nm (±3 nm) in the presence and absence of 5 mM $MgCl_2$ by using an Aminco-Chance split beam/dual wavelength spectrophotometer (Gross & Grenier, 1978). Red light (Corning CS 2-64, $\lambda > 650$ nm) was used as the actinic light source. Light intensities were measured by using a Kettering-Yellow Springs Instruments radiometer. Rates were calculated by using an extinction coefficient of 3.0 mM^{-1} cm^{-1} at 485 nm for DPCN (Shneyour & Avron, 1971).

Kinetics of P700 Oxidation and Reduction. The kinetics of P700 oxidation and reduction were determined by using an Aminco DW-2a split beam/dual wavelength spectrophotometer in its split beam mode of operation. The initial rate of P700 oxidation was measured as a decrease in absorbance at 430 nm (5-nm slit width) upon continuous illumination. Either a Baird Atomic 650-nm interference filter (half-bandwidth = 12 nm) or a Baird Atomic 710-nm interference filter (half-bandwidth = 12 nm) was used to isolate red actinic light. A Corning 4-96 filter and a Bausch & Lomb 90-1-620 interference filter were used to prevent actinic light from reaching the photomultiplier. The initial rate of P700 oxidation was determined as a function of light intensity by using an extinction coefficient for P700 of 45 mM^{-1} cm^{-1} at 430 nm (Ke, 1973). The initial rate of $P700^+$ reduction was measured as described by Gross (1979). Both artificial electron donors and the natural electron donor plastocyanin were used as sources of electrons to reduce oxidized P700 (see text for actual conditions). In all cases, oxygen served as the terminal electron acceptor.

Determination of Isoelectric Profile for Control and Modified PSI. Solutions of control and modified PSI containing 10 μg of chl/mL and 0.1% Triton X-100 were prepared. Three milliliters of each was placed in both a reference and sample cuvette. The pH of the PSI solution in the sample cuvette was adjusted in a stepwise fashion by using 0.1 M HCl or 0.1 M NaOH. The absorbance at 540 nm due to 180° light scattering was measured as a function of pH by using a Model 139 Hitachi Perkin-Elmer spectrophotometer.

Gel Filtration. Gel filtration was performed on control and modified PSI by using a 1.5 × 48 cm column of Bio-Gel P-300. One-half-milliter samples containing 75 μg of chlorophyll were applied to the column and eluted with a buffer containing 10 mM Tris–succinate, pH 7.0, 0.05% Triton X-100, and 1.0 M NaCl. Three-milliliter fractions were collected at a flow rate of 10 mL/h. The void volume and included volume of the column were determined with blue dextran and DNP-glycine, respectively.

Sodium chloride (1.0 M) was included in the elution buffer to prevent an ionic interaction that was found to occur between modified PSI and the column material in the absence of salt. This interaction presumably involved ionic attraction of cationic EDA-PSI (see Discussion) to a small number of carboxyl groups on Bio-Gel P-300 which are formed during gel preparation.

Chemicals. Ethylenediamine dihydrochloride and DCIP were obtained from Sigma Chemical Co. 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide was obtained from Pierce Chemical Co. Diphenylcarbazone and TMPD were obtained from Aldrich Chemical Co. The diphenylcarbazone was recrystallized once from methanol–water. Ascorbic acid was obtained from Fisher Scientific. Bio-Gel P-300 was purchased from Bio-Rad Laboratories. All other chemicals were reagent grade. Double-distilled, deionized water was used in all experiments.

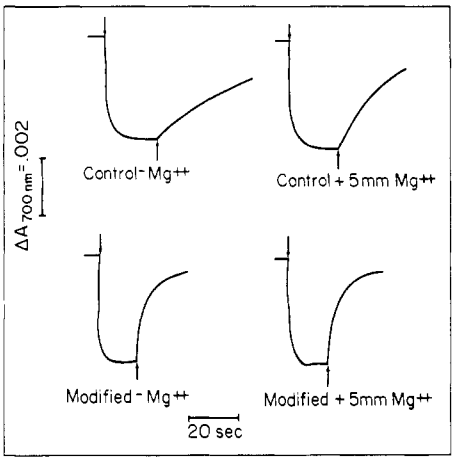

FIGURE 1: Comparison of the light-induced P700 assay for control and modified PSI. The assay mixture contained PSI (10 μg of chl/mL), 10 mM Tris-HCl, pH 8.2, and 6.7 mM ascorate in the presence and absence of 5 mM $MgCl_2$. The assay was performed as described under Experimental Procedures for measurement of $P700^+$ reduction.

Results

Initial Observations. When control PSI and PSI modified with ethylenediamine (EDA-PSI) were illuminated with high intensity blue light ($I = 4.7 \times 10^4$ ergs cm^{-2} s^{-1}), there was a rapid oxidation of all the P700 (Figure 1). Upon removal of the actinic light, the reduction of $P700^+$ in control PSI was very slow by using 6.7 mM ascorbate as the electron donor. Addition of 5 mM $MgCl_2$ to control PSI caused an increase in the rate of $P700^+$ reduction. This agrees with work on PSI by Gross (1979) and Lien & San Pietro (1979). The modification had no effect on the total amount of P700. However, the modification increased the rate of $P700^+$ reduction approximately 20-fold. Identical results were obtained for PSI modified in the presence and absence of 0.05% Triton X-100.

Determination of the Optimal Conditions for the Chemical Modification Reaction. The optimal reaction conditions described under Experimental Procedures were determined from a series of experiments which utilized the increase in $P700^+$ reduction by ascorbate as the assay (see the legend to Figure 1 for assay conditions). The ethylenediamine and EDC concentration dependences of the reaction were measured by using a series of reaction mixtures containing PSI (20 μg of chl/mL), a reaction pH of 6.0, and a reaction time of 1 h. For the ethylenediamine dependence, an EDC concentration of 0.1 M was present in each reaction. An inhibition of $P700^+$ reduction was found at ethylenediamine concentrations below 0.1 M. At ethylenediamine concentrations of 0.1 M or greater, a stimulation of $P700^+$ reduction was observed which saturated at 0.4 M ethylenediamine. Eighty percent of the observed stimulation occurred at 0.2 M ethylenediamine which was the concentration chosen for all experiments. A series of reactions were performed with 0.2 M ethylenediamine containing increasing amounts of EDC. The rate of $P700^+$ reduction increased as the amount of EDC present in the reaction was increased, with 0.1 M and 0.2 M EDC giving maximum rates. Above the concentration of 0.2 M EDC, the rate of $P700^+$ reduction was inhibited. Inhibition of $P700^+$ reduction was observed whenever the concentration of EDC exceeded that of ethylenediamine. Such inhibition was accompanied by the formation of large PSI aggregates.

This acid-catalyzed carbodiimide reaction was found to produce maximum rates of $P700^+$ reduction when the reaction was performed at pH 5 or 6. pH 6 was chosen for further modifications due to the fact that PSI has an isoelectric point near pH 5 (see Figure 2). When PSI was modified at pH 4, the rate of $P700^+$ reduction was inhibited, and large aggregates of PSI were formed similar to those observed under conditions where EDC was in excess of ethylenediamine.

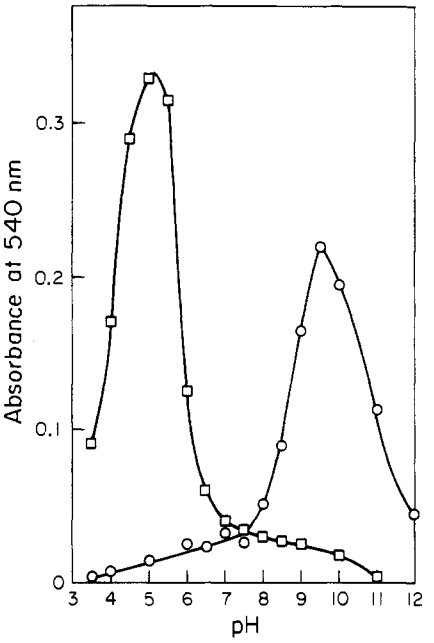

FIGURE 2: Determination of the isoelectric pH for control and modified PSI. Unbuffered solutions of control or modified were prepared which contained 10 μg of chl/mL and 0.1% Triton X-100. Both a reference and sample cuvette were filled with the PSI solution. The pH of the solution in the sample cuvette was slowly changed in a stepwise fashion with 0.1 M NaOH or 0.1 M HCl. Light scattering due to isoelectric precipitation of PSI was measured as a function of pH by measuring the absorbance at 540 nm where little chlorophyll absorption occurs. Control PSI (□). Modified PSI (○).

The modification reaction was found to be independent of the PSI concentration in the range between 10 and 80 μg of chl/mL. The reaction was complete after 60 min. The stimulated rate of $P700^+$ reduction in EDA-PSI was found to slowly decline after 14 days of storage at 4 °C. For this reason, all experiments were performed with EDA-PSI which had been prepared for less than 2 weeks.

Effect of Chemical Modification on the Isoelectric Point of the PSI Complex. For determination of whether the modification altered the net charge on the complex, turbidity (180° light scattering) was measured as a function of pH (Figure 2). The light scattering of the control was maximal at pH 5 due to isoelectric precipitation. This was shifted to pH 9.5 in the case of EDA-PSI, indicating a change in the net charge of PSI from negative to positive. Labeling experiments which show the incorporation of 600 ethylenediamine molecules into each PSI complex also support the idea of a generalized change in the charge on the complex rather than the specific incorporation of a few ethylenediamine molecules.

Effect of Chemical Modification on the Molecular Weight of the PSI Complex. In addition to a change in the net charge, it was possible that the chemical modification reaction caused cross-linking of polypeptides through amino and carboxyl groups on the protein. This cross-linking could have been intramolecular between the several polypeptides (Bengis & Nelson, 1975) within the complex or intermolecular between several intact complexes. Intramolecular cross-linking should not have occurred due to the high concentration of ethylenediamine used in the reaction. Excess ethylenediamine should prevent protein amino groups from competing with free

ethylenediamine as the nucleophile in the modification reaction.

Several lines of evidence suggest that intermolecular cross-linking of PSI monomers did not occur. First, the high concentrations of ethylenediamine (0.2 M) and low concentrations of PSI (20 μg of chl/mL) used in the modification reaction are factors which should have prevented intermolecular cross-linking. In addition, PSI modified in the presence of 0.05% Triton X-100, where PSI is known to exist as a monomer (Gross & Grenier, 1978), showed identical increases in the rate of $P700^+$ reduction by ascorbate compared to PSI modified in the absence of Triton X-100. Because stimulation of $P700^+$ reduction occurred in PSI modified in a monomeric state, intermolecular cross-linking of PSI monomers was not a requirement for the increased activity. The definitive experiment which argues against intermolecular cross-linking was gel filtration using Bio-Gel P-300 (see Experimental Procedures for conditions). Control PSI, EDA-PSI, and the stock solution of PSI from which the first two originated each eluted in the same fractions from the gel filtration column (data not shown). The identical molecular weight of PSI before and after chemical modification ruled out intermolecular cross-linking. These results indicate that the stimulation of $P700^+$ reduction by ascorbate is caused by a change in the charge of PSI and not by cross-linking of polypeptides.

Effect of Chemical Modification of the Activity of PSI. The activity of PSI particles can be divided into those processes that occur in the presence of light and those dark processes which are independent of light. The light processes include absorption of light, transfer of excitation energy from light-harvesting chlorophyll *a* molecules to the reaction center, and the primary photochemistry of P700. The dark processes involve the interaction of PSI with the electron donor as well as the dark electron-transfer steps between the donor and the terminal electron-acceptor oxygen.

The relationship between the rate of electron transport and light intensity can be expressed by eq 2 (Lumry & Spikes, 1959; Rieske et al., 1959) where v is the rate of electron transport and I the light intensity. K_L is the apparent rate constant for the light processes. K_D is the apparent rate of electron transport at infinite light intensity. Therefore changes in K_D indicate changes in those processes of PSI that are independent of light (i.e., dark processes). PSI electron transport was measured as a function of light intensity by using DPCN disproportionation (Vernon & Shaw, 1972; Shneyour & Avron, 1971). PSI particles with P700 inactivated by boiling for 5 min produced a measurable rate of DPCN disproportionation. This background rate increased linearly with light intensity from 0.5 to 1.5 μmol of DPCN (mg of chl)$^{-1}$ h^{-1} under the conditions of our measurements. Subtraction of this background rate from active PSI rates was performed before the calculation of K_D and K_L. The cause of this background rate may have been light-induced DPCN disproportionation by detergent-solubilized chlorophyll molecules as described by van Ginkel (1979). In our system, this chlorophyll-catalyzed rate was only 3–5% of the active PSI rates. This result is different from that of van Ginkel (1979) who found chlorophyll/Triton X-100 and PSI/Triton X-100 rates of DPCN disproportionation to be comparable and therefore concluded that the DPCN reaction was not specific for PSI. This difference in results may be due to the fact that van Ginkel used concentrations of DPCN which were 17 times higher than ours. This increase in DPCN concentration produced rates which were 3 orders of magnitude larger than the rates measured under our assay conditions. Perhaps DPCN disproportionation is not a specific PSI reaction at high DPCN concentrations. However, at the low DPCN concentrations used in our assay, approximately 95% of the measured rate of DPCN disproporationation was due to a PSI reaction.

$$1/v = 1/K_D + 1/(K_L I) \quad (2)$$

Table I: Effect of Modification of the Relative Quantum Yields for PSI Electron Transport Using Diphenylcarbazone Disproportionation[a]

	5 mM $MgCl_2$	$10^4 K_L$	% increase in K_L	K_D	% increase in K_D
control PSI	–	5.8 ± 0.8		27 ± 3	
control PSI	+	8.2 ± 1.5	41	41 ± 6	45
EDA-PSI	–	8.4 ± 0.7	45	100 ± 16	270

[a] Conditions are described under Experimental Procedures. Units for K_L are [μmol of DPCN (mg of chl)$^{-1}$ h^{-1}][ergs cm^{-2} s^{-1}]$^{-1}$. Units for K_D are μmol of DPCN (mg of chl)$^{-1}$ h^{-1}.

The results of the DPCN disproportionation are seen in Table I. The addition of $MgCl_2$ to control PSI caused a 41% increase in K_L. EDA-PSI also showed an equivalent increase in K_L. This suggests that binding of Mg^{2+} ions and the chemical modification reaction both stimulate the light processes of PSI. Mg^{2+} ions and chemical modification also produced an increase in K_D. The increase in K_D was larger for EDA-PSI than the cation stimulation of K_D in control PSI.

The light-induced P700 assay was used as a second independent method to compare effects of Mg^{2+} ions and chemical modification on the light and dark processes of PSI. The initial rate of P700 oxidation was measured under limiting light conditions as a function of light intensity. Under conditions of low light intensity, the initial rate of P700 oxidation should not be affected by the rate of $P700^+$ reduction and therefore can be used to measure the relative quantum yields. The relative quantum yield is proportional to the slope of the linear portion of a graph in which the rate of P700 oxidation is plotted as a function of light intensity.[2] Measurements were made by using interference filters to isolate actinic light at 650 and 710 nm. The 650-nm light is absorbed by light-harvesting chlorophyll *a* molecules in PSI while the 710-nm light is absorbed by the reaction center chlorophyll molecules. By examining rates of P700 oxidation under the two illumination conditions, we were able to determine if the stimulation of the light processes is caused by an increase in energy transfer from light-harvesting chlorophylls to the reaction center or an effect on the reaction center itself. Control PSI, EDA-PSI, and the stock solution of PSI from which the first two originated are compared in Figure 3. The stock PSI was included in order to determine if the incubation of PSI at pH 6.0 affected the control PSI. The slope of the curve for control PSI is less than that for stock PSI under 650-nm illumination conditions. Thus incubation at pH 6.0 inhibited the energy transfer processes of PSI. This may have been due to isoelectric precipitation which begins at pH 6.0 (see Figure 2). On the other hand, the slope of the curve for EDA-PSI is greater than that for stock PSI when 650-nm light was used for illumination. When 710-nm light is used, the slope for control PSI, stock PSI, and EDA-PSI is approximately the same. These results indicate that a primary effect of chemical modification is to increase energy transfer.

The initial rate of $P700^+$ reduction can be analyzed by Michaelis–Menton kinetics [see Gross (1979)] which gives two parameters for comparison of divalent cation and chemical

[2] The relative quantum yield (ϕ) is defined by the formula $V_0 = \phi I$ where V_0 is the rate of P700 oxidation and I is the light intensity.

Table II: Effect of Modification of Electron Donation to P700$^+$ [a]

electron donor	constant	control PSI		EDA-PSI	
		$-MgCl_2$	+5 mM $MgCl_2$	$MgCl_2$	+5 mM $MgCl_2$
ascorbate	K_m [b]	278 ± 12	88 ± 5	3.5 ± 0.1	7.6 ± 0.3
	V_{max} [c]	31 ± 18	18 ± 3	22 ± 1	20 ± 4
DCIP	K_m	7.9 ± 0.3	16.5 ± 0.6	1.9 ± 0.1	2.8 ± 0.1
	V_{max}	17 ± 3	111 ± 52	90 ± 10	67 ± 13
TMPD	K_m	135 ± 6	200 ± 6	123 ± 3	201 ± 10
	V_{max}	120 ± 33	193 ± 77	110 ± 18	179 ± 77
plastocyanin	K_m	no reac-	32.6 ± 1.2	1.3 ± 0.1	2.0 ± 0.1
	V_{max}	tion	263 ± 130	130 ± 56	289 ± 103

[a] The initial rate of P700$^+$ reduction was measured as described under Experimental Procedures. The assay mixture contained control PSI or EDA-PSI (10 μg of chl/mL) and 10 mM Tris-HCl, pH 8.2. The assay mixture was titrated with increasing concentrations of electron donor and the initial rate of P700$^+$ reduction measured after each addition. When DCIP, TMPD, and plastocyanin were used as electron donors, ascorbate was included in the assay mixture at a concentration of 50 μM to keep these electron donors reduced. The background rate of P700$^+$ reduction by 50 μM ascorbate was subtracted from the measured rates. Double-reciprocal analysis was used to analyze the data. A least-squares error calculation was performed to determine the error in K_m and V_{max} to within one standard deviation. [b] Units for K_m are mM for ascorbate and μM for all other donors. [c] Units for V_{max} are μmol of P700$^+$ (mg of chl)$^{-1}$ h^{-1}.

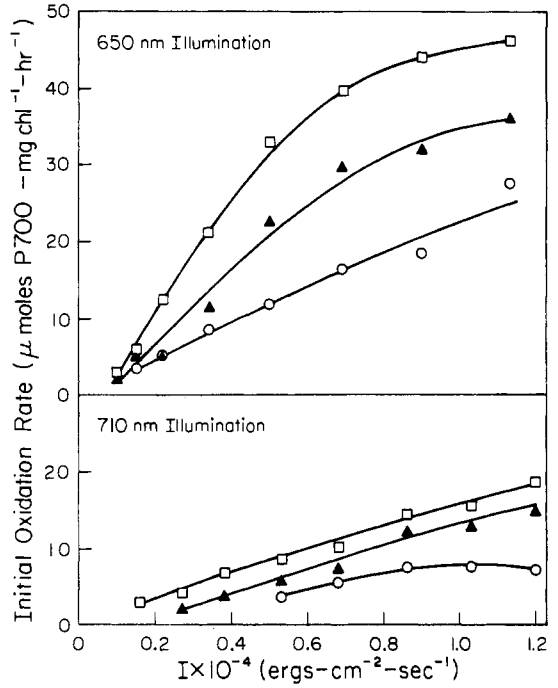

FIGURE 3: Effect of chemical modification on the light-induced oxidation of P700. The initial rate of P700 oxidation was measured as a function of light intensity as described under Experimental Procedures. The intensity of the actinic light was varied by varying the voltage supplied to the light source. Light intensities were measured by using a Kettering-Yellow Springs Instruments radiometer. The assay mixture contained PSI (20 μg of chl/mL), 5 mM Tris-HCl, pH 8.2, 2 mM ascorbate, and 0.33 μM DCIP. Modified PSI (□). Control PSI (○). Stock solution of PSI (▲).

modification effects on the dark processes of PSI.[3] The apparent K_m is a measure of the binding of the electron donor to PSI while the V_{max} reflects the environment of the electron transfer from the donor to P700$^+$. Table II summarizes the effect of Mg^{2+} ions and chemical modification on the K_m and V_{max} for several electron donors.

Both chemical modification and $MgCl_2$ alter the K_m's for the various electron donors in a way which can be explained on the basis of the screening of the surface charges on the PSI complex. For example, $MgCl_2$ decreased the K_m for the interaction of ascorbate with the negatively charged control PSI complex. The results are consistent with those of Itoh (1979) who found that addition of cations to broken chloroplast membranes caused an increase in the rate of P700$^+$ reduction by negatively charged electron donors. The increase in the rate was found to be the result of the lowering of the surface potential of the negatively charged membranes, making the electron donor more accessible to P700.

Conversely, salts should increase the K_m for the interaction of positively charged electron donors with control PSI by screening favorable electrostatic interactions. The increase in the K_m for TMPD observed upon addition to $MgCl_2$ to control PSI agrees with these predictions.

Chemical modification which alters the net charge on the PSI complex should also decrease the K_m for negatively charged electron donors. This was observed for both DCIP and ascorbate. The predicted increase in the K_m for TMPD was not observed, however.

The effects of salts on the modified EDA-PSI should be opposite to those on the control PSI (i.e., they should increase the K_m for negatively charged electron donors and decrease the K_m for positively charged electron donors). $MgCl_2$ increased the K_m for ascorbate and DCIP as predicted. However, the effect on the K_m for TMPD is opposite to that predicted.

Addition of $MgCl_2$ and chemical modification also increased the V_{max} for DCIP. $MgCl_2$ also increased the V_{max} for TMPD, although there is some question about the statistical significance of these results. However, Gross (1979) did observe a statistically significant increase in the V_{max} for both ascorbate and TMPD after addition of $MgCl_2$.

These effects can also be based on the screening of or alteration of the surface charge on the complex. In this case, the change in charge would cause a conformational change in the protein which would, in turn, increase the V_{max}. In support of this argument, a Mg^{2+}-induced conformational change has been observed in PSI particles (Gross & Grenier, 1978).

The most significant alteration of P700$^+$ reduction by chemical modification involved electron donation by plasto-

[3] Under continuous illumination, high concentrations of electron donors produce a steady-state rate of P700$^+$ reduction which competes with the steady-state rate of P700 oxidation. This results in a decrease in the total P700 signal. Such a decrease in signal results in an underestimate of the true rate of P700$^+$ reduction. The rate of P700$^+$ reduction (V) is $V = k[P700_{ox}]$, where k is a rate constant (dependent on the electron donor concentration, Mg^{2+} concentration, etc.) and $[P700_{ox}]$ is the concentration of oxidized P700. When the P700 is totally oxidized, V is proportional to k. If the P700 is not completely oxidized, the observed rate will be less than the true rate. We have corrected for this according to $V' = V_{obsd}P700_{total}/P700_{ox}$, where V' is the corrected rate, V_{obsd} is the observed rate, $P700_{total}$ is the maximum P700 signal measured at low donor concentrations, and $P700_{ox}$ is the observed P700 signal.

cyanin. Plastocyanin is a small copper protein which serves as the electron donor to $P700^+$ in vivo as well as in isolated PSI particles (Boutler et al., 1977). Table II shows that there is an absolute requirement of divalent cations for the interaction of plastocyanin with control PSI particles. The addition of Mg^{2+} allows high rates of $P700^+$ reduction in control PSI. Chemical modification of PSI completely abolishes the Mg^{2+} requirement. Compared to control PSI in the presence of Mg^{2+} ions, EDA-PSI has a much lower K_m for plastocyanin. This agrees with the idea that Mg^{2+} and chemical modification make the PSI surface more positive which allows the negatively charged plastocyanin molecule a better access to its binding site on the PSI surface. Addition of Mg^{2+} to EDA-PSI caused an increase in the K_m for plastocyanin, indicating that divalent cations inhibit the binding of plastocyanin to EDA-PSI. This would be expected because salts would screen the favorable electrostatic interactions between the negatively charged plastocyanin and the positively charged EDA-PSI. The addition of Mg^{2+} to EDA-PSI also produced an increase in the absolute value of V_{max} for plastocyanin. Because the high rates of $P700^+$ reduction by plastocyanin produced a large error in the extrapolated values of V_{max}, it is difficult to state that the changes in V_{max} are significant.

Discussion and Conclusions

The ability of PSI to harvest light and interact with electron donors is altered by chemical modification of the complex using a water-soluble carbodiimide and ethylenediamine. The modification produces a change in the net charge on PSI as the result of negatively charged protein carboxyl groups being replaced by the positively charged free amino group of ethylenediamine (see eq 1). Three lines of evidence suggest that this change in charge is caused by incorporation of a large number of ethylenediamine molecules and not by the modification of a few specific amino acid residues. First, high concentrations of ethylenediamine (0.2 M) and EDC (0.1 M) were found to be optimal for the modification reaction. If only a few specific amino acids were involved, low optimal concentrations of modifying reagents would be expected. Second, the EDA-PSI has an isoelectric pH of 9.5 compared to 5.0 for control PSI. A large percentage of the carboxyl groups would have to be modified to convert an anionic protein into a cationic protein. Third, labeling experiments with [^{14}C]-ethylenediamine indicate 600 molecules of ethylenediamine are incorporated for each reaction center. Although the reaction of EDC with protein carboxyl groups is the only pathway that leads to ethylenediamine incorporation, there are two other amino acids which can react with an EDC molecule. These are cysteine (Carraway & Triplett, 1970) and tyrosine (Carraway & Koshland, 1968). The isourea derivatives produced by the reaction of these amino acids with EDC contain a (dimethylamino)propyl group from the original carbodiimide molecule. These tertiary amines would also contribute positive charges to EDA-PSI.

The labeling experiments also gave some insight into the isolated PSI comples. The molecular weight of a PSI monomer is estimated to be 100–200 kdaltons (Thronber et al., 1977; Gross & Grenier, 1978). The combined mole percentage of aspartic and glutamic acid residues in PSI has been estimated to be in the range of 15% (Thornber et al., 1977; P. M. Abdella and E. L. Gross, unpublished results). By use of the upper limits for the molecular weight of PSI and the mole percentage of amino acids with carboxyl group side chains, the calculated value for the number of carboxyl groups associated with each reaction center is approximately 250. This value is lower than the value of 600 mol of ethylenediamine incorporated per mol of reaction center. Two possibilities exist which would explain why the amount of ethylenediamine incorporated is larger than the potential number of reactive carboxyl groups on PSI. First, perhaps a portion of the reaction centers are inactivated upon isolation. However, if this were true, more than 50% of the isolated reaction centers would be inactivated to account for the [^{14}C]ethylenediamine results. A more feasible explanation was suggested by Thornber et al. (1977). They described two types of PSI monomers which are of the same molecular weight but differ in that one contains the P700 reaction center while the other does not. The presence of a large amount of PSI complex which is not photoactive would explain the large number of carboxyl groups modified for each reaction center.

The reaction of EDC with PSI in the absence of available nucleophile inhibits the activity of PSI. When 0.1 M EDC is incubated with PSI at pH 6.0, the PSI particles form large aggregates. Aggregation is accompanied by inhibition of both energy transfer from light-harvesting chlorophyll *a* molecules to the reaction center and electron donation to $P700^+$. This type of aggregation and its effects are observed under conditions where EDC is in excess of the ethylenediamine. The drop in the rate of $P700^+$ reduction when PSI is modified at pH 4.0 is also associated with aggregation of PSI. This illustrates another way to effectively create an excess of EDC by performing the modification at a pH where very little unprotonated amine is present to act as a nucleophile. This was examined by comparing ethylenediamine and glycine ethyl ester which have amine p*K*'s of 7.5 and 7.75, respectively, with ethanolamine and 2-aminoethanesulfonic acid which have amine p*K*'s of 9.5 and 9.0. The reaction of PSI with EDC was performed at pH 6.0 in the presence of these amines. Aggregation of PSI occurred for the two compounds with amine p*K*'s of 9.0 or above. No aggregation occurred for ethylenediamine or glycine ethyl ester which have p*K*'s much closer to the reaction pH. The conclusion is that aggregation of PSI occurs when EDC is in excess by either adding more carbodiimide than nucleophile or using amines which are not good nucleophiles at the reaction pH. The nature of EDC-promoted PSI aggregation is unknown. Protein cross-linking and some type of noncovalent protein interaction are potential causes. It is known that 1% sodium dodecyl sulfate does not break up the aggregation.

Gross & Grenier (1978) demonstrated that the addition of Mg^{2+} ions to PSI produced a conformational change in the complex which resulted in an increase in energy transfer from light-harvesting chlorophyll *a* to P700. We find that EDA-PSI also exhibits an increase in energy transfer. The conclusion is that binding of Mg^{2+} ions to PSI and chemical modification of PSI with ethylenediamine produce a PSI complex in a more positively charged environment. This cationic environment creates a PSI conformation that results in an increase in energy transfer.

The dark processes of PSI were found to be regulated by a combination of surface potential and protein conformation, both of which are regulated by charges on the PSI surface. Gross (1979) found that Mg^{2+} ions increase the rate of electron transfer from artificial electron donors to $P700^+$. We find that both Mg^{2+} and chemical modification of PSI increase the rate of electron transfer from DCIP and TMPD to $P700^+$. These V_{max} effects are regulated by protein conformation. A more favorable conformation for electron transfer exists when Mg^{2+} ions bind to PSI carboxyl groups or when the carboxyl groups are replaced by amino groups through covalent attachment of ethylenediamine molecules. The binding of electron donors to PSI is affected by surface potential. Itoh (1979) found this

type of regulation for electron donation to $P700^+$ in broken chloroplast membranes. In our system, the K_m values reflect the surface potential of isolated PSI. If Mg^{2+} ions and chemical modification lower the surface potential, this should appear as a decrease in K_m for the negatively charged donors ascorbate and DCIP and an increase in K_m for the positively charged donor TMPD. The K_m values determined from our experiments support surface potential regulation of donor binding with two exceptions. Mg^{2+} should lower the K_m for DCIP while chemical modification should increase the K_m for TMPD. We did not observe this. Therefore other factors such as protein conformation play a role in binding of electron donors to PSI.

Divalent cations also play an important role in regulating electron transfer from plastocyanin to $P700^+$ by regulating surface charge. Tamura et al. (1980) and Haehnel et al. (1980) have shown that plastocyanin donation to P700 in chloroplast membranes is stimulated when cations are present due to a decrease in membrane surface potential which allows plastocyanin more accessibility to P700. We find an absolute requirement for divalent cations in order for plastocyanin to be an electron donor to isolated PSI. Similar results were observed by Lien & San Pietro (1979). This increase was later found to be the result of lowering the K_m for plastocyanin binding (Davis et al., 1980). Chemical modification of PSI substitutes completely for divalent cations. In fact, EDA-PSI has a lower K_m for plastocyanin binding than the K_m measured when Mg^{2+} ions are added to control PSI. This suggests that permanently replacing anionic charges with cationic groups at the site of PSI/plastocyanin interaction is more effective than charge shielding by divalent cations. These results are consistent with the surface potential model for the interaction of spinach plastocyanin with PSI, which are both negatively charged proteins.

PSI made cationic by chemical modification mimics the activity of PSI to which Mg^{2+} ions have been added. Both processes increase energy transfer from light-harvesting chlorophyll *a* to P700 as well as regulate electron donation to $P700^+$. Thus divalent cations regulate PSI activity by altering the net charge of the PSI environment as the result of cation binding to protein carboxyl groups. Alteration of ionic environments by cations has been implicated in regulation of grana stacking and chlorophyll fluorescence (Barber et al., 1977; Rubin & Barber, 1980) and donation to $P700^+$ by artificial electron donors (Itoh, 1979) and plastocyanin (Tamura et al., 1980f Haehnel et al., 1980) in chloroplast membranes. They define a role for surface charge regulation for these processes at the thylakoid membrane surface. We have now presented results which implicate surface charge in regulation within the individual photosystems.

Acknowledgments

We thank Dr. D. J. Davis and Dr. A. San Pietro for the generous gift of a portion of the plastocyanin used in this work.

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