Redox titration of fluorescence yield of photosystem II

(photosynthesis/primary electron acceptor/photophosphorylation)

BACON KE, FRED. M. HAWKIDGE*, and SAURA SAHU†

Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387

Communicated by Martin D. Kamen, April 5, 1976

ABSTRACT. The variable fluorescence yield of photosystem II is dependent on the redox state of the fluorescence quencher molecule or the primary electron acceptor of the system. We have carried out redox titrations of fluorescence yield of a photochemically active photosystem-II reaction-center particle and have measured the redox potential of the photosystem-II primary acceptor.

During reductive titrations using dithionite as the reductant, only a single quenching transition was observed. For instance, at pH 7.0, the midpoint potential of the fluorescence transition is -325 mV, and those at a pH between 6.0 and 7.5 are consistent with a pH dependence of about 80 mV/pH unit. At a given pH, the midpoint potential of the transition closely corresponds to that of the most negative transition previously measured in unfractionated chloroplasts (both by chemical reductive titration). Oxidative titrations using ferricyanide as the oxidant yielded hysteresis in the titration curves.

Similar changes in fluorescence yield were observed in redox titrations by electrochemical reduction or oxidation. Electrochemical reductive and oxidative titrations yielded reversible transitions, contrary to the hysteresis observed during chemical oxidative titration. From coulometric-titration data, we have estimated that most likely one electron is involved in the redox transition of the fluorescence-quencher or primary-electron-acceptor molecule of photosystem II. These findings are consistent with the current proposal that a membrane-bound plastoquinone functions as the primary acceptor of photosystem II.

The photosynthetic apparatus in green plants consists of two photosystems. Photon capture by each photosystem results in an energetically excited state leading to the formation of two oppositely charged species. The primary electron donors in both photosystems are probably made up of chlorophyll a molecules present in a specialized environment. Whereas the primary donor of photosystem I, P-700, has been known for some time (1), the primary donor of photosystem II, P-680 (2, 3), and the primary electron acceptor of photosystem I, P-430 (4), have been discovered only more recently.

The primary electron acceptor of photosystem II, because of its role in controlling the variable fluorescence of photosystem II, was first designated by Duysens and Sweers (5) as "Q," which stands for the quencher of fluorescence. The quencher molecule or the primary acceptor of photosystem II has often been thought to be a plastoquinone (6), as exogenous quinones have been found to quench strongly the chlorophyll in chloroplasts and algae (7). The primary-acceptor plastoquinone, however, must possess some unique properties different from plastoquinone molecules in the secondary-acceptor pool (3).

Steinh and Witt (8) observed a spectral species, "X-320", whose kinetic behavior was said to be consistent with that of the photosystem-II primary acceptor. Knaff and Arnon (9) observed another spectral species, "C-550", which also appeared to have properties of the photosystem-II primary acceptor (10). More recently, van Gorkom (11) and Pulles et al. (12) identified plastoquinone as the primary acceptor of photosystem II. Both groups found that the light-induced absorption changes associated with the photosystem-II primary acceptor apparently also included absorption changes that have previously been attributed to both X-320 (8) and C-550 (9, 10).

The redox potential of the photosystem-II primary electron acceptor was first measured by Kok et al. (13), who monitored the fluorescence-yield changes as a function of the redox potential, and they reported a midpoint potential of +180 mV. Similar fluorescence titration of spinach chloroplasts by Cramer and Butler (14) showed quenching occurred at -35 mV and -270 mV (at pH 7), and the midpoint potentials were pH-dependent. The titration data could be interpreted as to represent either two quencher molecules, each with a different redox potential, or a single quencher molecule that undergoes a two-stage reduction. The authors favored the less negative potential as the representative value for the primary electron acceptor of photosystem II, but the significance of the lower potential quencher remained undecided. Similar redox potential values of the photosystem-II primary acceptor have also been estimated by titrating the absorption changes of C-550 (10, 15). From an indirect estimate of the pK value of the photosystem-II primary acceptor, Knaff (16) recently suggested that the acceptor may function with an effective potential of -130 mV.

We report here data on the redox titration of fluorescence yield from a photochemically active photosystem-II reaction-center particle isolated from spinach chloroplasts by Triton fractionation (17-19). We have found that in all cases examined only one quenching transition occurred and, at a given pH, the midpoint potential of the transition closely corresponds to that of the most negative transition previously found in unfractionated chloroplasts (14). From coulometric-titration data we have estimated that most likely one electron is involved in the redox transition of the quencher molecule.

EXPERIMENTAL

The photosystem-II reaction-center particles were prepared from spinach chloroplasts by Triton fractionation using the method described previously (17, 18). Chloroplasts used for duplicating the fluorescence titrations were prepared in the same way as described in ref. 14. All chemical were of reagent grade when available and were used without further purification.

Chemical titrations using dithionite (Fluka, purum grade) as the reductant and ferricyanide as the oxidant were carried out in a special 1 cm × 1 cm quartz cell described in detail recently (20, 21). The redox mediator concentrations are given in the figure legends.
Electrochemical titrations were carried out in the electrochemical cell of Hawkridge and Kuwana (22). The cell was modified by replacing the tin-oxide window with a quartz plate, and a gold-foil electrode was fixed along the side wall of the cell. This cell has a path length of 12 mm and a total volume of 1.75 ml. The chloroplast sample containing the redox mediators was deoxygenated in a separate reservoir bulb attached to the cell before it was transferred into the cell. A potentiostat assembled from instrument modules [McKee Pedersen Instruments (MPI), Danville, Calif.] or a PAR model 173 potentiostat (Princeton Applied Research, Princeton, N.J.) was used for controlling the reduction or oxidation.

The fluorescence was measured with a weak monitoring beam (600 nm, 8-10 ergs/cm²-sec (8-10 mW/m²), isolated by a 10-nm wide interference filter (Baird Atomic), and modulated by an electro-optic modulator at 25 kHz (23)). The modulated fluorescence passing through Corning filters 2–64 and 4–77 (cut-on at 687 nm) was detected by an EMI 9558 photomultiplier tube and processed through a PAR model 211 preamplifier and a PAR model 220 Lock-In amplifier and recorded on a chart recorder. When the 1 cm × 1 cm quartz cell was used in the chemical titrations, the fluorescence emitted at right angle to the measuring beam was detected when the electrochemical cell (22) was used, the fluorescence emitted in line with the measuring beam was detected from the backside of the cell.

RESULTS

Potentiometric titration of fluorescence yield using a chemical reductant or oxidant

Fluorescence induction in the photosystem-II particles has been examined previously (19) and correlated with their photochemical activities as a further confirmation of their photosystem-II character. The internal oxidant pool of photosystem II was titrated by light quanta and estimated from the fluorescence-rise curves (19). The redox properties such as the oxidation-reduction potential can be determined by a potentiometric titration of the fluorescence yield (13, 14). The inference derived from both light and chemical titrations is based on the interpretation of Duysens and Sweers (5), namely, the fluorescence yield is dependent on the redox state of the primary electron acceptor or the fluorescence quencher of photosystem II.

The photosystem-II particle may contain only components immediately associated with the reaction center. A potentiometric titration could therefore yield a change less complicated than in chloroplasts, allowing more insight concerning the redox components in photosystem II. Fig. 1 shows the results of a potentiometric titration of the photosystem-II particles, using a monitoring beam at about 10 ergs/cm²-sec (10 mW/m²). The titration was carried out at four pH values from 6.0 to 7.5, at half a pH-unit intervals, and from +100 to −400 mV (all potentials expressed relative to the normal hydrogen electrode unless otherwise indicated); the negative potential limit was restricted by the prevailing pH of the medium. The magnitude of fluorescence yield appears to depend on pH. This behavior, although its nature is unknown, parallels the pH dependency of the photochemical activity of these particles (24).

In the reductive titration (from right to left in the figure), a single, relatively simple change occurred at all pH values. For instance, at pH 7.0, the midpoint potential is −325 mV, and those at other pH values are consistent with a pH dependency of about 60 mV/pH unit. However, the slope of the titration curves suggests that the reaction may involve more than two electrons. The oxidative titration represented by the dashed curves going from left to right in the figure are much less well defined either in the pH dependency of the midpoint potentials or in the slopes of the curves.

The courses of titration observed previously for chloroplasts are quite different from those observed here: for instance, at pH 7, two major breaks were observed during the reductive titration, one at a much more positive potential of −20 mV, and the other at −320 mV (14). Similarly, a hysteresis was observed during the oxidative titrations, i.e., the titration curves were shifted toward a more positive potential (14) similar to that shown in Fig. 1, but the breaks were reasonably well defined.

In view of the above-mentioned difference, we carried out a potentiometric titration of the fluorescence yield with unfraccionated chloroplasts, and the results are shown in Fig. 2. At pH 7.0, a reductive titration produced three transitions, at midpoint potentials of +10, −235, and −321 mV. The first transition was always the largest at all three pH values examined, and its slope appeared to correspond to a one-electron change. The second transition was relatively small. The third transition, occurring at the most negative potential, was of intermediate size and its slope would suggest a redox transition involving more than two electrons at all pH values examined. The transitions show a shift of 60 mV/pH unit. Hysteresis also occurred during the oxidative titrations (cf. ref. 14). Interestingly, the most negative transitions observed during the reductive titration of chloroplasts at all three pH values correspond closely with the single transitions observed in the photosystem-II particles, both in the midpoint potentials as well as the slope of the transitions.
Potentiometric titration of fluorescence yield by electrochemical reduction or oxidation

We further used electrochemical titrations to examine the fluorescence yield changes. The electrochemical behavior of the mediators in the presence of chloroplasts or subchloroplast particles was first examined, prior to fluorescence yield measurements by recording a cyclic voltammogram of the sample system in the same electrochemical cell with which fluorescence yield was to be measured. Fig. 3 shows typical cyclic voltammograms for samples containing unfraccionated chloroplasts (at pH 7.0) and photosystem-II particles (at pH 7.5), with the appropriate redox mediators present. The cyclic voltammograms show the expected reversible reductions and oxidations of the mediators occurred in the presence of (sub)chloroplasts when the voltage was scanned in the negative and positive directions, respectively.

After the cyclic voltammetric check, the potential of the gold-foil electrode was stepped to −900 mV (versus the Ag/AgCl (1.0 M KCl) reference electrode) and held constant at that potential by the potentiostat; the fluorescence yield was recorded as a function of the accumulated charge injected into the sample system. A typical recording is shown in Fig. 4 for the fluorescence yield change of the photosystem-II particles at pH 7.0. In our present experimental arrangement, the actual potential of the system at any given time was measured by an additional platinum electrode placed in the cell. When the potential of the platinum electrode was measured versus the reference electrode, all other leads were momentarily disconnected from the potentiostat.

In the titration shown in Fig. 4, the fluorescence yield of the photosystem-II particle remained at a low level up to −540 mV (versus Ag/AgCl), then increased steadily and became saturated near −500 mV. A slight drop in fluorescence yield was apparent even when the potential was maintained constant. However, when the applied potential was stepped back to 0 mV (versus Ag/AgCl) to oxidize the system, a sharp drop in fluorescence yield occurred. The fluorescence-yield increase during the first cycle occurred over a potential span of approximately 50 mV, while the fluorescence-yield decrease and subsequent fluorescence increases and decreases all occurred with an even steeper slope (see Fig. 4). This was probably caused by a trace of oxygen present in the sample initially.

The midpoint potentials estimated from the point-by-point measurements during electrochemical titrations were consistently more negative than those obtained from the continuously recorded values of the chemical titrations. The reason for this difference is not known. However, steep slopes in the fluorescence transitions were obtained both by chemical (Fig. 1) and by electrochemical (Fig. 4) titrations, and the pH dependence of the midpoint potentials appears to be the same, i.e., about 60 mV per pH unit. On the other hand, the reverse titration by electrochemical oxidation was markedly different from those observed in the chemical titrations. Whereas the chemical titrations always showed hysteresis during the reverse oxidative reaction, as shown in Figs. 1 and 2, and also reported previously by Cramer and Butler (14), electrochemical oxidative titrations appear to be reversible and free of such hysteresis at all pH values we have examined. It is possible that the oxidative titrant, ferricyanide, being a well-known electron acceptor for pho-
tosystem II, may not be able to effect a controlled oxidation of the fragments through the mediators.

**Coulometric titration of fluorescence yield**

Because both the chemical and electrochemical titrations yielded the unusual slope, which suggests that the redox transition of the quencher or primary-acceptor molecule involves more than two electrons, we attempted to estimate the number of electron changes more directly by coulometric titrations in the modified electrochemical cell (22).

Since the course of the fluorescence rise is quite well defined and the onset of the fluorescence rise is sharp, the coulometric measurement was made by stepping the applied potential to $-900$ mV (versus Ag/AgCl). Immediately before the onset of fluorescence rise, simultaneous recordings of the charge injected and the electrolysis time were begun and the actual potentials of the system at the beginning and end of the fluorescence rise were noted. Typical results of such a titration of the photosystem-II particle at pH 7.5 are shown in Fig. 5.

![Fluorescence Intensity (relative)](image)

**Fig. 5.** Coulometric titration of fluorescence yield of the photosystem-II subchloroplast particles in the modified electrochemical cell (22). Sample composition same as in legend of Fig. 3. See text for detailed discussion.

The charge and time measurements were started at the potential of $-446$ mV (versus Ag/AgCl) and ended at $-605$ mV. The total charge was determined by passing the electrolysis current flowing through the working electrode first through a current-to-voltage converter and then integrating this voltage with respect to time with a chopper-stabilized operational amplifier (MPI 1031 unit). The total charge consumption accompanying the fluorescence rise was estimated from the distance between the two intercepts of the curve with the extrapolations of the initial and final portions of the curve (dashed lines). During the electrolysis, nonfaradaic events also consuming charges must be accounted for and corrected from the total charge. The nonfaradaic current was separately estimated by running the same experiment in the absence of the redox mediators. The net charge was estimated to be 0.64 micro-equivalent from three reductive titrations and 0.60 micro-equivalent from three oxidative titrations. The sample containing the photosystem-II particle had a chlorophyll concentration of 17 $\mu$M. As the ratio of the concentration of the reaction-center particles (C-S50 and P-680, as well as cytochrome $b_{559}$) to the total chlorophyll in this sample was previously estimated to be $\frac{1}{10}$ (19), the primary-acceptor concentration was assumed to be the same, which would be 0.42 $\mu$M. The number of electron equivalent per molecule is then 1.4–1.5. Comparable results were also obtained from a coulometric titration of the photosystem-II particle at pH 6.5 in a thin-path length cell designed by Heineman and coworkers (25). It should be noted that the photosystem-II fragments, though highly purified, most likely also contain other components that may also undergo redox changes in the fluorescence transition region. Thus, the net electron charge measured here may not be accounted for exclusively by the fluorescence change. With this consideration, we would estimate that the redox change of the quencher molecule most likely involves one electron.

**DISCUSSION**

Given the fact that only a single redox transition was apparent in the fluorescence yield change in the photosystem-II reaction-center particles at any given pH, and that the transition agrees well with that observed at the most negative potential in unfractionated chloroplasts, we are led to conclude that the more negative transition is representative of the quencher or primary-electron acceptor molecule of photosystem II. The more positive transitions observed in chloroplasts probably represent the secondary electron acceptors that are in a redox equilibrium with the primary acceptor in the unfractionated chloroplasts. The photosystem-II subchloroplasts presumably are largely devoid of secondary acceptors (18, 19).

The general view at present is that (bound) plastoquinone is functioning as the primary electron acceptor of photosystem II (11, 12). Adopting this view and accepting the basic view that the fluorescence yield change is a valid indication of the redox state of the photosystem-II primary acceptor (5), despite numerous recent findings that the photosystem-II fluorescence yield can also be quenched by a number of other components (3), we will now examine some implications of our present findings.

Although it is generally accepted at present that light-induced charge separation in photosystem II results in a very strong oxidant and a relatively weak reductant, the red photon absorbed by photosystem II, however, has sufficient energy to produce a more negative reductant than currently envisioned. In protic solvents, one-electron reduction of quinones is not easily observed, because the semiquinone radicals readily disproportionate into a fully reduced hydroquinone and a fully oxidized quinone. The redox potential of such quinone reactions has been reported to be near 100 mV (26). However, in aprotic solvents, semiquinones are very stable species. Furthermore, the redox potential of the one-electron reduction of quinones in aprotic solvents is at a much more negative value, ranging from $-500$ to $-500$ mV (27). Thus, the relatively negative redox potentials observed for the fluorescence yield increase attributed to the quencher or primary-acceptor molecule of photosystem II is consistent with the notion that the plastoquinone molecules, which are presumably tightly bound to the photosynthetic membrane, are located in a hydrophobic environment.

As the intersystem potential span, i.e., the potential difference between the oxidized P-700 and the reduced primary acceptor of photosystem II, is vital for photosynthetic ATP formation, the more negative redox potential observed for the system-II quencher molecule may lead to a reassessment of the energetic aspect of photophosphorylation (2, 16).

Stiehl and Witt (28) found that plastoquinone in the secondary-acceptor pool was reduced to the hydroquinone form even though supposedly only one electron was transferred through the electron-transport chain when single turnover short flashes were used for excitation. This anomaly was explained by assuming that the system-II primary acceptor (designated as X) is present as a "twin," i.e., $X\times X$, and each $X$ is complicated with a primary-donor molecule (say, $P-690$). The photochemical reaction leads to a doubly charged twin, $X\times X\times ^{-}$, which reduces two molecules in the plastoquinone (PQ) pool, and two plasto-semiquinone molecules in turn disproportionate to one PQ and PQ$^{2-}$. The half-life of the primary acceptor X was
found to correspond to the risetime of plastoquinone reduction (27).

Our observation that reduction of the system-II quencher molecule involves one electron appears to be consistent with the "twin" quencher scheme. Although each quencher molecule undergoes a one-electron reduction, the close association of two such quencher molecules in a twin unit could lead to complex reaction kinetics and conceivably account for the unusual slopes of the fluorescence-yield titration curves.

We thank Dr. W. R. Heineman for many useful discussions regarding the use of the thin-layer electrochemical cell and Dr. P. Mehta for helpful comments on the paper. We also thank Dr. Michael J. Brown of the Imperial Chemical Industries for a gift sample of the 1,1'-trimethylene-2,2'-dipyridyld dibromide and Mr. E. R. Shaw for providing the photosystem-II particle. This work was supported by a National Science Foundation Grant GB-29161. This is contribution no. 550 from Charles F. Kettering Research Laboratory.