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POTENTIOMETRIC TITRATION OF THE FLUORESCENCE YIELD OF SPINACH CHLOROPLASTS

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SUMMARY

The fluorescence yield of spinach chloroplasts was measured as a function of oxidation–reduction potential under anaerobic conditions at pH 6.0, 7.0, and 8.0. The potentiometric-titration curve showed two fluorescence quenching processes, both quenching in the oxidized state. The complete titration curve could be reasonably fit with a Nernst equation for the sum of two one-electron-transfer reactions with different midpoint potentials. At pH 7.0 the two transitions had midpoint potentials of about −35 mV and −270 mV and both showed a pH dependence of about −60 mV per pH unit. The hypothetical quencher, Q, assumed to be responsible for light-induced fluorescence-yield changes in green photosynthetic systems could be ascribed to the more positive quenching component.

INTRODUCTION

Much of the data on chlorophyll fluorescence-yield changes in green plants can be explained in terms of a hypothetical component, Q, which acts as the primary electron acceptor for Photosystem 2[1,2]. The oxidized state of Q presumably quenches chlorophyll fluorescence by acting as a trap for the energy absorbed by the bulk of the chlorophyll in Photosystem 2 while the reduced state, QH, does not act as a trap and therefore does not quench. The redox nature of the quenching process was indicated by experiments in which a high fluorescence yield of a leaf, maintained under anaerobic conditions, was markedly decreased on the introduction of air[3] and by an increase of fluorescence yield obtained on adding dithionite to chloroplasts[2,4].

KOK *et al.*[4] attempted to determine the redox potential of Q from measurements of the fluorescence yield as a function of the electrochemical potential of the medium. They reported a midpoint potential of +180 mV for the fluorescence-quenching process in chloroplast particles from Scenedesmus mutant No. 8. However, we found that addition of ascorbate to chloroplasts produced no change of fluorescence yield even though Q should have been reduced completely if its midpoint potential were

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E_{m7}, midpoint potential at pH 7.0; E_m (1) and E_m (2), midpoints of high and low potential quenching components.

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+180 mV. We have repeated the potentiometric titration of fluorescence yield with spinach chloroplasts with particular precautions taken to exclude O_2 and to maintain adequate redox buffering to allow the chloroplasts to equilibrate with the redox environment.

EXPERIMENTAL PROCEDURE

Chloroplasts were prepared from locally grown spinach. 50 g of depetioled leaves were ground in 150 ml of ice-cold 0.4 M sucrose, 0.05 M Tris–HCl (pH 7.8), 0.01 M NaCl, and 0.25 g ascorbic acid for 15 sec in a Waring Blendor. The preparation was filtered through a layer of muslin and centrifuged alternately at low speed (300 $\times$ *g*, 1200 $\times$ *g* for the final spin) for two cycles. The chloroplast pellet, containing about 5 mg of chlorophyll, was homogenized in a tissue grinder.

The chloroplasts were resuspended at 7–10 μg chlorophyll per ml in 13 ml of a medium consisting of 0.4 M sucrose, 0.1 M phosphate buffer (pH 6.0 or 7.0) or 0.1 M Tricine (pH 8.0), and 4 mM $MgCl_2$ at room temperature (22°). After it was established that the major changes in fluorescence yield occurred in the potential region between zero and —300 mV at pH 7.0, the following set of redox buffers were added to the reaction medium to provide continuous buffering over this potential range: 30 μM neutral red (E_{m7} = —325 mV); 0.1 mM anthraquinone 2-sulfonate (E_{m7} = —225 mV); 30 μM 2-hydroxy-1,4-napthoquinone (E_{m7} = —145 mV); 10 μM indigotetrasulfonic acid (E_{m7} = —46 mV); 30 μM 5-hydroxy-1,4-napthoquinone (E_{m7} = + 33 mV); 30 μM 1,4-napthoquinone (E_{m7} = +60 mV). The midpoint potentials for these compounds are given by CLARK[5], and we have measured very similar values under the conditions of our experiment. The choice of the appropriate redox buffers and their concentrations were dictated by several criteria: adequate buffering capacity in the desired potential region; absence of appreciable absorption of either the exciting or the emitted light; absence of fluorescence in the red region of the spectrum; and absence of anomalous interaction with the chloroplasts, such as fluorescence quenching independent of redox potential.

The design of the titration vessel and gas train was patterned after that of CUSANOVICH *et al.*[6] with the exceptions that only a single cuvette was used, the argon gas used for deaeration was further purified by passage through a $Cr_2(SO_4)_3$ solution and the gas train to the cuvette was all glass. The titration was performed in a 1-cm cuvette sealed to a 24/25 outer standard taper joint. A combination platinum Ag–AgCl electrode (Instrumentation Lab. 15020), or the platinum electrode and a combination pH electrode (IL 14040), together with syringe needles for gas delivery and exhaust were inserted into the cuvette through a silicone rubber stopper. Thus, redox potential and pH could be measured at the same time as fluorescence intensity.

The potential of the Ag–AgCl (saturated KCl solution) reference electrode was calibrated against a saturated quinhydrone electrode and found to be 197 mV. This value was used to convert the potential measurements to the scale based on the hydrogen electrode.

The reaction medium containing redox buffers was deaerated for at least 2 h before the titration with argon gas (Liquid Carbonic, prepurified, $<$ 5 ppm O_2) which had been passed through a manganese oxide column and a wash bottle filled with $Cr_2(SO_4)_3$ to further reduce the O_2 content of the argon. The gas was bubbled through

the cuvette during the titration with a small amount of antifoam (Dow Corning AF) added to minimize foaming. About 1 mg of glucose oxidase (Mann, specific activity about 170 units) and 13 mmoles glucose (0.01 M final concn.) were added to the cuvette to scavenge any residual O_2 or photosynthetically produced O_2. The reductant and oxidant were, respectively, 0.01 M $Na_2S_4O_6$ (in 0.01 M NaOH) and 0.01 M $Fe(CN)_6^{3-}$, which had been boiled and deaerated before addition to the cuvette. Aliquots of 1–10 μl of the oxidant and reductant were added with a gas-tight microsyringe (Hamilton, N1705) through a septum in a sidearm. The titration experiments were generally begun at the low potential end to further scavenge any traces of O_2.

Potentiometric titrations at low potential are very sensitive to small amounts of O_2. We were not able to obtain reproducible results below 0 V at pH 7.0 until we used an all-glass gas-flow system and added the $Cr_2(SO_4)_3$ scrubber. A test titration of 30 μM FMN at pH 7.0 was entirely reversible and gave an excellent fit to the theoretical curve for a two-electron transition obeying the Nernst equation with a midpoint potential of —205 mV. A similar test titration with 30 μM neutral red ($E_{m7} = -325$ mV) showed an effective midpoint potential of —320 mV but there was a discrepancy between the reductive and oxidative titrations at potentials below —330 mV. In the measurements with chloroplasts, the potential measurements were often uncertain at potentials below about —275 mV, perhaps because of small amounts of O_2 evolved photosynthetically in the weak exciting light which were not completely eliminated by the glucose–glucose oxidase.

Fluorescence was excited with a very weak (3 ergs·cm^{-2}·sec^{-1}) chopped (300 cycles/sec) monochromatic (660 nm) beam and the emission of wavelengths longer than 680 nm was measured with an EMI 9558C phototube (blocked with Corning filters 9830 and 2030), connected to a PAR Lock-In amplifier. Fluorescence intensity was recorded as a function of time. Approx. 1 min was required for the potential and fluorescence to stabilize after the addition of an aliquot of oxidant or reductant. In the experiment in which the fluorescence-yield change due to actinic irradiation was compared to that due to chemical reduction, the intensity of the measuring beam was increased to 100 ergs·cm^{-2}·sec^{-1}. The fluorescence excited by the steady actinic beam ($7 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} at 653 nm) was not detected by the Lock-In amplifier which responded only to fluorescence excited by the chopped measuring beam.

RESULTS

The fluorescence yield of spinach chloroplasts measured as a function of redox potential under anaerobic conditions at pH 7.0 is shown in Fig. 1. Each point represents the intensity of fluorescence obtained after stabilization of the suspension to the potential indicated. The suspension, initially at low potential (< —300 mV), was oxidized by sequential additions of small aliquots of $Fe(CN)_6^{3-}$ until the minimal fluorescence was obtained. The reverse reductive titration was then performed by sequential additions of small aliquots of $S_4O_6^{2-}$. The dashed curves represent an attempt to fit the experimental data with theoretical curves based on the Nernst equation for two one-electron redox reactions with different midpoint potentials. The two components of the theoretical curve for the oxidative titration in Fig. 1 have midpoint potentials of —35 mV and —270 mV and contribute 47.5 and 52.5%, respectively, to the total amplitude. The theoretical curve for the reductive titration assumed

two components with midpoint potentials of —20 mV (40 %) and —320 mV (60 %). The oxidative titration has been extended to +500 mV at pH 7.0 with no further change in the fluorescence yield.

The ratio of the maximum fluorescence yield observed at the start of the oxidative titration to the minimum yield was 6.2:1 in the experiment shown in Fig. 1. The ratio

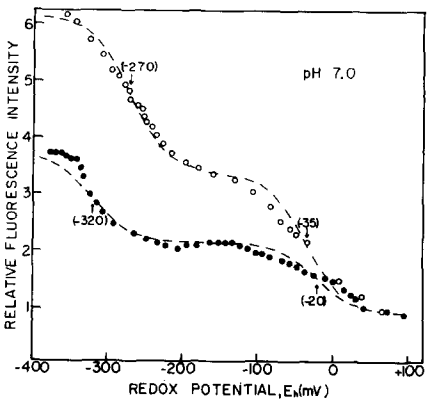

Fig. 1. Relative fluorescence intensity of chloroplasts at pH 7.0 as a function of the oxidation–reduction potential of the medium. Open circles: oxidative titration starting at low potential by sequential additions of small aliquots of 0.01 M $Fe(CN)_6^{3-}$. Solid circles: subsequent reductive titration by sequential additions of aliquots of 0.01 M $S_4O_6^{2-}$. Dashed curves: theoretical curves based on the Nernst equation for two one-electron transitions with different midpoint potentials, $E_m(1)$ and $E_m(2)$. For the oxidative titration, $E_m(1) = -35$ mV contributing 47.5 % to the total amplitude and $E_m(2) = -270$ mV contributing 52.5 %; for the reductive titration, $E_m(1) = -20$ mV, 47 %, and $E_m(2) = -320$ mV, 55 %.

of the fluorescence yield at the plateau region of the titration (—100 mV to —200 mV) to the minimum yield was 3.5:1. These ratios vary somewhat from experiment to experiment. The largest ratio of maximum to minimum fluorescence that has been observed is 9:1 with a plateau about midway between the two extremes.

Determination of the titration curves in the presence of 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) gave the same results, indicating that the photochemical fluorescence quencher equilibrated with the redox potential of the medium directly rather than through one or more subsequent members of the electron-transport chain. At the low intensity of light (3 ergs·cm^{-2}·sec^{-1}) used for fluorescence excitation, addition of DCMU caused the minimum fluorescence yield to increase only 10 %.

An aspect of these titrations which does not appear in Fig. 1 was the presence of a large pool of endogenous reductant which slowly reduced the added $Fe(CN)_6^{3-}$ and made it difficult to equilibrate and hold potentials more positive than about —50 mV at pH 7.0. A pool of reductant with similar properties has been observed in the photosynthetic bacteria[6,7].

The shape of the fluorescence-yield titration curves of Fig. 1 is not attributable to any property of the redox dyes. This was checked by control experiments in which the redox buffers were either left out of the medium, one by one, or replaced by another if one of suitable potential and color could be found. In such control experiments, the two components of the titration curve could always be seen. The oxidation

of the indigotetrasulfonic acid (10 μM) caused a decrease in the fluorescence intensity of about 3% due to increased absorption of the 660-nm exciting light. The color change at this wavelength of the neutral red (30 μM) and the substituted naptho-quinones used as redox buffers was completely negligible, as was their fluorescence, under the conditions of the experiment.

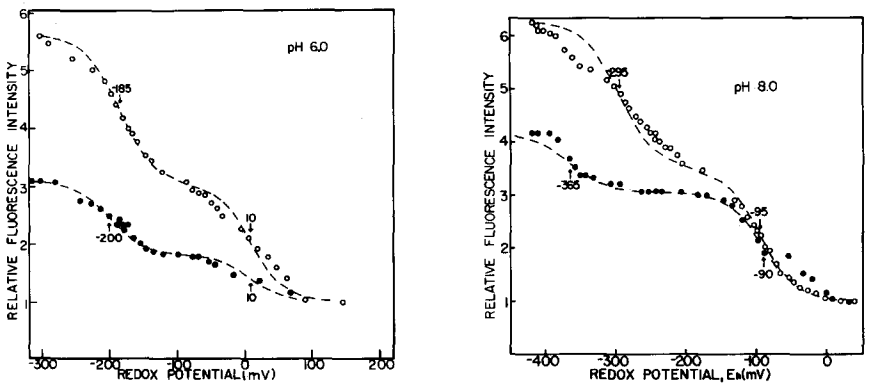

Fig. 2. Same as Fig. 1 but at pH 6.0. For the oxidative titration, $E_m(1) = +10$ mV, 45%, and $E_m(2) = -185$ mV, 55%; for the reductive titration, $E_m(1) = +10$ mV, 39.5%, and $E_m(2) = -200$ mV, 60.5%.

Fig. 3. Same as Fig. 1 but at pH 8.0. For the oxidative titration, $E_m(1) = -95$ mV, 47.5%, and $E_m(2) = -295$ mV, 52.5%; for the reductive titration, $E_m(1) = -90$ mV, 65%, and $E_m(2) = -365$ mV, 35%.

The loss of fluorescence which occurred over a period of 2–3 h between the beginning of the oxidative titration and the end of the reductive titration appeared to be due in part to a loss of chloroplasts from suspension and, in part, to a gradual decrease of yield during the periods at low potential. The slight foaming action during the continuous bubbling caused the chloroplasts to adsorb to the glass surfaces of the electrodes and cuvette. This was minimized but not eliminated by the antifoam. The effect of low potential on fluorescence yield may have been due to a deleterious effect of $S_4O_6^{2-}$, but dithiothreitol could not be used as an alternative reductant because it acted too slowly at low potentials.

The effect of pH on the potentials of the fluorescence-yield changes is shown in Figs. 2 and 3. At pH 6.0 (Fig. 2) the midpoint of the high-potential transition, as determined by the curve fitting, was +10 mV for both the oxidative and reductive titrations and the midpoints for the low potential transition were, respectively, —185 mV and —200 mV. At pH 8.0 (Fig. 3) the midpoints were —95 and —90 mV for the high-potential transition, and —295 and —365 mV for the change at low potential, although the two latter values are somewhat uncertain because of the poor redox buffering at these low potentials. The dependence of the midpoint potentials on pH, summarized in Table I, appears to be about —60 mV per unit change in pH.

A comparison of the fluorescence-yield-change obtained with actinic light to that obtained with $S_4O_6^{2-}$ under aerobic conditions at pH 7.0 is shown in Fig. 4. In this experiment the intensity of the chopped measuring light was increased to 100 ergs·cm^{-2}·sec^{-1} in order to permit measurements in the presence of the strong steady

TABLE I

MIDPOINT POTENTIALS OF HIGH, $E_m(1)$, AND LOW, $E_m(2)$, POTENTIAL QUENCHING COMPONENTS AS A FUNCTION OF pH

	Titration	*pH of chloroplast suspension*	*Midpoint potentials (mV)*		
			6.0	*7.0*	*8.0*
$E_m(1)$	Oxidative		+10	−35	−95
	Reductive		+10	−20	−90
$E_m(2)$	Oxidative		−185	−270	−295
	Reductive		−200	−320	−365

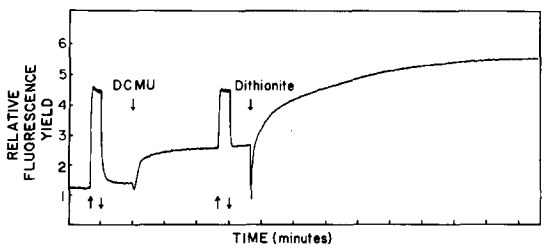

Fig. 4. Relative fluorescence yield of chloroplasts at pH 7.0 under aerobic conditions. Actinic light (653 nm, $7 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}) on and off at upward and downward arrows. DCMU (1 μM) and $S_4O_6^{2-}$ (crystals) added where indicated. The minimum fluorescence yield is one on the relative fluorescence-yield scale (based on an estimate that the measuring beam at 100 ergs·cm^{-2}·sec^{-1} increased the fluorescence yield by 20%).

actinic beam. The fluorescence yield in this intensity of measuring light was about 20% greater than the minimum yield. Irradiation with 653-nm actinic light at $7 \cdot 10^4$ ergs·cm^{-2}·sec^{-1} gave the maximum light-induced fluorescence-yield increase which was about 4.5 times the minimum yield (after correction for the effect of the measuring light). Irradiation in the presence of 1 μM DCMU gave the same maximum yield. Addition of $S_4O_6^{2-}$, however, increased the fluorescence yield still further to about 5.5 times the minimum yield. Under anaerobic conditions, we would have expected an even greater increase on addition of $S_4O_6^{2-}$.

DISCUSSION

The potentiometric titration of fluorescence yield indicated two redox processes or components which quench fluorescence in the oxidized state. Previous work on the kinetics of the light-induced fluorescence-yield increase indicated that a single redox component, Q, quenched chlorophyll fluorescence in the oxidized, but not the reduced state[1]. The magnitude of the light-induced fluorescence-yield change in Fig. 4 suggests a tentative identification of the photochemical quencher with the more positive component observed in the potentiometric titration of fluorescence. The additional yield over the maximum light-induced yield obtained by adding $S_4O_6^{2-}$ to the chloroplasts may be ascribed to the low potential component.

Any detailed comparison between the light-induced fluorescence-yield changes in Fig. 4 and the titration curves is tenuous because of the variations of fluorescence yield between different chloroplast preparations and because of the possible deleterious

effects of $S_4O_6^{2-}$ on the fluorescence yield in the titration experiments. The observation that the light-induced fluorescence yield (Fig. 4) was greater than the yield at the titration plateau (Fig. 1) suggests that the low potential component might be partially reduced photochemically by the chloroplasts. However, the more conservative conclusion would appear to be that there is one photochemical quencher which is the more positive component in the titration curve. Preliminary experiments designed to elucidate the function of the low potential component by separating the two quenchers into Photosystem 1 and Photosystem 2 activities (by differences in fluorescence emission spectrum or by fluorescence measurements on digitonin particles) have been negative.

The fluorescence yield may be taken as a measure of the reducing power of Photosystem 2. During sustained electron transport in the presence of an electron acceptor, the fluorescence yield is 50–100 % above the minimum level which would correspond to a potential of about 0 V at pH 7.0. In the absence of an electron acceptor, the fluorescence yield during irradiation is 3–5 times the minimum yield, indicating potentials of —100 mV or less. At pH 8.0, where chloroplast reactions are often assayed, the potentials would be even lower.

The titration curves for both components indicate a one-electron process with a pH dependence of —60 mV per pH unit. The chemical reduction of either quenching component can be written as:

$$Q + e + H^+ \rightleftharpoons QH$$

The photochemical reduction of Q which results in the light-induced fluorescence-yield increase does not necessarily involve the photochemical transfer of a proton or require an equilibration between Q and the protons in solution within the lifetime of the excited state of the reaction center. It seems more reasonable to suggest a photochemical transfer of an electron to give Q^- which subsequently takes a proton from the environment.

For simplicity, we have assumed that the titration curves indicated two independent quenching processes. However, we have not ruled out the possibility of one quenching compound Q with two states of reduction, QH and QH_2, with different midpoint potentials. The latter case would require that fluorescence quenching by QH be intermediate between that of Q and QH_2.

Kok *et al.*[4] previously titrated the fluorescence yield of cell-free particles of Scenedesmus mutant No. 8 potentiometrically at pH 7.5. They reported that the midpoint potential of the quencher was +180 mV and that the redox reaction was a one-electron process which was independent of pH. Our results suggest a midpoint potential of —35 mV at pH 7.0 for the photochemically active quenching component and a one-electron transfer which is pH dependent. One of the main consequences of these results is that the potential drop in the electron-transport chain between Photosystem 2 (—35 mV) and Photosystem 1 (+430 mV)[8] is sufficient for a coupled phosphorylation reaction.

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