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Photosystem I generates a free-energy change of 0.7 electron volts or less

Angel R. Arcelay [a], Robert T. Ross [a] and Bilal M. Ezzeddine [b]

[a] *Department of Biochemistry and* [b] *Bio-Medical Engineering Program, Ohio State University, Columbus, OH (U.S.A.)*

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The intensity of delayed luminescence from wild-type *Scenedesmus obliquus* was used to refine a previous determination (Marchiarullo, M.A. and Ross, R.T. (1981) Biochim. Biophys. Acta 636, 254–257) of the free-energy change in Photosystem II, which we find to be 0.99 eV under physiological conditions. Similar measurements were made of the very weak luminescence from a mutant deficient in plastoquinone A, originally with the expectation that this luminescence would be from Photosystem I. However, the action spectrum for excitation of mutant luminescence is that of Photosystem II. There is no measurable change in the properties of the mutant luminescence with changes in excitation wavelength extending to 708 nm, leading us to conclude that Photosystem I contributes less than 5% of the emission 10 ms after excitation. This limit on the intensity of emission from Photosystem I places an upper limit of 0.69 eV on its free-energy difference.

Introduction

Diagrams of the photosynthetic electron transport chain often position its components according to their standard, or midpoint, electrode potentials [1]. In such a diagram, the free-energy input from a quantum of light is represented as the difference between the standard potentials of the primary donor and the primary acceptor [2].

The redox properties of the donor and acceptor sides of PS I [3] are better known than those of PS II [4]. However, little is known about the actual working potentials of these donors and acceptors, which are likely to differ from the standard potentials by 0.10 V or more. As a consequence, we have been interested in an alternative method of determining the free energy captured by PS I.

The free-energy difference between the ground and an excited state of any photochemical system, at any instant, is a known function of the intensity of luminescence emitted by the excited state. Thus, measuring the luminescence intensity allows one to compute the free-energy difference of any system at any time [5].

In the photosynthetic apparatus, back reaction of the energy storage pathway of PS II leads to luminescence which can be measured for hours after the cessation of illumination (reviewed by Govindjee and Jursinic [6] and Jursinic [7]). If one assumes that most delayed luminescence emitted by the photosynthetic apparatus is from a homogeneous population of Photosystem II centers, then direct measurement of the intensity of luminescence, with appropriate calibrations, permits simple evaluation of the free-energy difference. With this assumption, we have previously

Abbreviations: PS I, Photosystem I; PS II, Photosystem II.

Correspondence: R.T. Ross, Department of Biochemistry, Ohio State University, 484 West 12th Avenue, Columbus, OH 43210, U.S.A.

found that PS II of the green alga *Scenedesmus quadricauda* has a free-energy difference of 1.0 eV when illuminated by light in the physiological range of intensities [5].

In this paper, we present work from mutant No. 11 of *S. obliquus*, deficient in plastoquinone A, whose very weak luminescence we have used to place an upper limit on the amount of free energy generated by Photosystem I. We measured the luminescence from 7 ms to 2.5 s after cessation of the actinic light.

The first report of possible PS I luminescence was by Bertsch et al. [8], using the same *Scenedesmus* mutant. Under their experimental conditions, the intensity of luminescence from the mutant was 250-times less than that from wild-type algae, suggesting that PS I luminescence is at most several hundred times weaker than PS II luminescence. Most other attempts to see PS I luminescence in vivo have found it to be below the limits of detection [9,10], although there have been occasional claims that PS I luminescence is visible in vivo under particular circumstances [11–13].

Despite the difficulty of seeing PS I luminescence in vivo, it has been reported from isolated particles. Shuvalov [14] reported emission with a lifetime of 20 ms. Sonneveld and collaborators [15] measured a component of luminescence in the microsecond range associated with recombination from P-700$^+$ and reduced acceptor A$^-$.

Theory

The emission properties of an excited electronic state are usually independent of how it was created. This is because the lifetime of most molecular excited states is sufficiently long that there is time for all the substates to reach equilibrium prior to emission.

Although excited-state equilibration is the usual expectation, there is occasionally incomplete equilibration of aromatic molecules. Most often this is because the energies of the electronic states depend on interactions with surrounding solvent which do not equilibrate within the fluorescence lifetime. The lack of equilibration is observed as a change in emission spectrum or emission kinetics upon excitation at the long-wavelength absorption edge, or as a change in emission spectrum with time following illumination (reviewed by Lakowicz [16]).

For the purpose of the remainder of this section, we shall assume that equilibration is achieved. If this assumption is correct, then the excited state has the well-defined thermodynamic properties characteristic of all equilibrated systems, such as free energy. Furthermore, since both the ground state and excited state have internal equilibrium, the free-energy difference between them must be independent of the path joining them.

From this reasoning, the free energy provided by the light-driven step of a photochemical process is equal to the free energy of the non-radiative process

$$\mathrm{P} \rightleftharpoons \mathrm{P}^* \tag{1}$$

where P and P* are the ground and excited states of the pigment. This free-energy change is given by the Nernst equation as

$$\Delta G = \Delta G^\circ + k_B T \ln([\mathrm{P}^*]/[\mathrm{P}]) \tag{2}$$

The radiative transition from P* to P is accompanied by luminescence, with the intensity of this emission proportional to the concentration [P*]. Thus, the free energy of the transition varies as $k_B T$ ln (luminescence intensity).

Using a similar but more formal and general line of reasoning [5,17–19], one can establish a relationship between the intensity of luminescence and the free energy of the light-driven transition. This relationship requires only equilibration within the electronic states for its validity.

The general equation for the free-energy change is simply [5]:

$$\Delta G = k_B T \ln(R_{\mathrm{lum}}/R_{\mathrm{therm}}) \tag{3}$$

where R_{lum} is the rate of luminescence, and R_{therm} is the rate of transitions occurring, in either direction, due to absorption of thermal black-body radiation,

$$R_{\mathrm{therm}} = \int I_{\mathrm{bb}}(\lambda)\sigma(\lambda)\,\mathrm{d}\lambda \tag{4}$$

where $\sigma(\lambda)$ is the absorption cross-section at λ,

and $I_{bb}(\lambda)$ is the intensity of radiation emitted by a black-body at the ambient temperature:

$$I_{bb}(\lambda) = 8\pi n^2 c\lambda^{-4}[\exp(hc/\lambda k_B T) - 1]^{-1} \quad (5)$$

If the sample does not absorb and emit light equally in all directions, $\sigma(\lambda)$ must be averaged over solid angle and polarization.

Accurate measurements of absolute $\sigma(\lambda)$ can be difficult or not possible, but ΔG can still be evaluated accurately using the following set of equations. First, we note that for any specimen in a particular instrument,

$$Y = \eta R_{lum} \quad (6)$$

where Y is the luminescence signal recorded by the instrument and η is a measure of the sensitivity of the instrument. Second, we note that for a photoluminescent specimen, one which luminesces as a direct consequence of illumination, the rate of luminescence may be written as

$$R_{lum} = \phi_{lum} \int I_{ext}(\lambda)\sigma(\lambda)\, d\lambda \quad (7)$$

where ϕ_{lum} is the quantum yield of luminescence, and I_{ext} is the intensity of incident illumination. Combining Eqns. 6 and 7, one may then in principle evaluate η for an instrument by using a reference specimen having a known quantum yield. However, this evaluation need not be done explicitly. Rather, Eqn. 6 may be applied to both the sample (s) having unknown ΔG and to the reference (r) having known quantum yield. Then, substituting in Eqn. 4 for the sample and Eqn. 7 for the reference, we find that

$$\frac{R_{lum}}{R_{therm}} = \phi^s_{lum} \frac{Y^s \int I_{ext}\, \sigma^r\, d\lambda}{Y^r \int I_{bb}\, \sigma^s\, d\lambda} \quad (8)$$

for the sample, which may then be used in Eqn. 3 to find ΔG. If the sample and reference have the same geometry, any quantity proportional to $\sigma(\lambda)$ may be used in its place. In optically thin specimens, absorbance is proportional to cross-section, so that absorbance may be used in Eqn. 8.

A material with high fluorescence quantum yield may be used as the reference, so there is no experimental difficulty in using a reference specimen which is optically thin at all wavelengths of interest. However, with a sample having very weak luminescence, one may need a high optical absorbance in order to get an accurately measurable signal. Fortunately, the conflict between high optical absorbance for good sensitivity and low optical absorbance for accurate use of absorbance in Eqn. 8 is not so great as it may at first appear, because most of the integral of Eqn. 4 applies to wavelengths at which the absorbance is relatively low.

Through the Kennard-Stepanov relationship [20,17] the integrand of Eqn. 4 is also the emission spectrum.

$$\sigma(\lambda) I_{bb}(\lambda) = F(\lambda) \quad (9)$$

where $F(\lambda)$ is the emission in relative quanta per second per wavelength interval. By definition, this integrand is largest at the wavelength of the emission peak. The left side of Eqn. 9 is evaluated from a short wavelength, at which its value has become quite small, out to the longest wavelength at which the absorbance can be measured. The right side of the equation is evaluated over the wavelength range in which emission can be measured. A fit of these two curves, weighted for the relative reliability of individual points, determines the best scaling of the right side and the best estimate of the integrand. The resulting value of R_{therm} will have an error of about 20% for a typical aromatic molecule, with a consequent error in ΔG of 0.2 $k_B T$ [19].

Eqns. 2–4 hold both in the presence and in the absence of external illumination. The intensity of emission during illumination, or during the first few nanoseconds following illumination, can be used to determine the ΔG between the ground and excited state of pigments in the presence of actinic light. 1 μs or more after any actinic illumination has ceased, any detectable pigment luminescence must be from excited states which themselves have a much longer lifetime than the several nanoseconds of a typical singlet state, or which have been populated from long-lived states.

Any photochemical system with high energy-conversion efficiency must emit luminescence from

excited pigments which have been repopulated by reversal of the energy storage pathway [18]. At a time Δt after illumination, luminescence is due primarily to back reaction of energy-storage intermediates which have pseudo-first-order reverse rate constants near to $1/\Delta t$. The intensity of this luminescence is thus a fairly accurate measure of the ΔG between chemical intermediates which are a 'distance' Δt on either side of the light-driven step.

In a thermodynamically ideal photochemical energy converter, all energy-storage intermediates derived from the excited state are in equilibrium with the excited state, so that the population of the excited state is held constant, with a consequent constant luminescence, until the concentration of these storage intermediates changes. However, previous measurements [5,9] of luminescence from PS II have shown that the intensity of luminescence a few μs after illumination is about 200-times less than the intensity of the luminescence during illumination, showing that fairly early energy-storage intermediates are about k_BT ln $200 = 0.14$ eV out of equilibrium with the average free energy of the pigments responsible for fluorescence during illumination. Part of this drop occurs between the antenna pigments and the reaction center, and is due to the finite rate of energy transfer to the reaction center compared to the rate of fluorescence from the antenna. The rest of the drop appears to occur between the reaction center and intermediates which appear in nanoseconds, and may be due to a loss pathway such as the tunneling of a radical-pair intermediate back to the ground state.

There are two major issues confronting us as we attempt to apply the theory just presented to Photosystem I. First, does the excited state of this photosystem have the equilibrium required for application of the theory? Second, how do we resolve PS I emission in the presence of stronger PS II emission?

Attempting to answer both of these questions, Marchiarullo and Ross [21] used an algebraic technique known as factor analysis or principal components analysis to resolve mathematically the prompt fluorescence excitation and emission spectra of PS I and PS II in intact systems. The spectra obtained by this technique should be similar, if not identical, to the spectra of delayed luminescence from the same photosystems, and may thus be used as reference spectra in the search for delayed emission from PS I. Application of the Stepanov relationship to the spectra obtained by factor analysis indicated that PS II is at, or very near to, thermal equilibrium at the ambient temperature and also suggested that PS I may not be well-equilibrated. Despite some concern about the quality of equilibration in PS I, we will proceed because no other method for evaluating the free energy is available.

Materials and Methods

Normal and mutant cultures of the algae *S. obliquus* were purchased from The Culture Collection of Algae at the University of Texas. The wild-type (UTEX 393; known as Gaffron's D-3, from Emerson's laboratory) was grown in a modified Kessler's medium [22], with continuous illumination from a bank of fluorescent lights (General Electric, cool white) at an intensity of 1 W/m^2 at 26°C. Mutant No. 11 (UTEX 2016) produced by Bishop [23], and deficient in plastoquinone A [24], was grown heterotrophically in the dark with the Kessler's medium supplemented by 0.5% glucose and 0.025% yeast extract [25]. Cells used for experiments were harvested in their logarithmic phase, pelleted, washed twice, and suspended in fresh media with 0.5% methyl cellulose added to inhibit settling during luminescence measurements.

Illumination was provided by a 100 W tungsten-halogen lamp, with wavelength selection obtained using a 3.2 cm water filter followed by colored-glass and interference filters. Most measurements used Corning 3-67, 4-96 and 4-97 colored-glass filters and a Ditric 580 nm short-pass interference filter; this actinic light had peak intensity at 564 nm with a half-bandwidth of 24 nm. Excitation intensity was controlled with two circular neutral density wedges (Kodak).

An integrating sphere was used to illuminate the samples with maximum uniformity and to collect emission efficiently. This sphere was 25 mm in diameter, with left and right halves machined out of matching blocks of poly(tetrafluoroethylene) (Teflon). The assembled sphere had

three apertures: one at the top, 10 mm in diameter, to hold the sample tube, and two side apertures of 6 mm each, at right angles, facing the center of the optical paths for excitation and emission.

The 1.0 ml samples were held centered on the vertical axis of the sphere as a cylinder of 8×23 mm, in a sample tube made of 10 mm quartz tubing. Samples had an absorbance of 0.15–0.20 cm^{-1} at 680 nm, measured in the scattered transmission accessory of a Cary 118 spectrometer, with apparent absorbance at 800 nm subtracted to correct for residual scattering loss.

During luminescence measurements, the intensity of the exciting light was determined with a photodiode (Hamamatsu S1226-5BQ, connected to a Keithley 610C Electrometer) attached to the side of the integrating sphere. The spectral sensitivity of the photodiode was calibrated by Mark Tirpack [26]. The relationship between photodiode readings and total effective light intensity on the sample tube was determined with chemical actinometry, using Reinecke's salt [27].

Light emitted by the sample was collected with $f/0.7$ optics through a Corning 2-60 colored-glass filter. This filter has an absorbance of 1 below 625 nm and was used to reduce the amount of luminescence from parts of the instrument itself which reached the detector. The emission was measured with a photon-counting photomultiplier tube (Hamamatsu R943-02) cooled to $-20\,°C$ to reduce the dark-counting rate to 5–15 s^{-1}. The absolute efficiency of the detection system was determined using cresyl violet as a fluorescent standard of known quantum yield [28]. Photon-counting pulses from the PMT were stored in a microcomputer (Commodore-64) in 250 bins, each of width 10 ms.

Both the excitation and emission optical paths were equipped with an electro-mechanical shutter (Uniblitz, Vincent Assoc.). The sample was subjected to alternating periods of 6.0 s with illumination and 4.0 s darkness. Samples were adapted to this light regime for 10 min before the start of data collection, which then continued for several hundred cycles. The emission shutter was opened just after the excitation shutter was closed, and was then closed for a 1.0 s measurement of background counting rate prior to the next period of illumination. Data collection began 7 ms after the excitation shutter was half-closed; a small spurious signal, due mostly to luminescence from glass parts of the instrument, was observable for an additional 50 ms.

Action spectra for excitation were obtained using interference filters having bandwidths of 9–12 nm for half-maximum transmittance. For each filter, the luminescence was observed at three or more different excitation intensities. Interpolation on a log–log plot of emission intensity vs. excitation intensity was use to identify the excitation intensity producing the emission intensity chosen as the criterion for equal action.

The relative shape of emission spectra were compared by measuring intensity using only the Corning 2-60 filter, and with the addition of Schott colored-glass filters having cutoff wavelengths of 695 and 715 nm.

Results

Fig. 1 shows the intensity of delayed luminescence as a function of exciting light intensity. The top two curves show the behavior of the wild-type at two different average delay times after the shutter was closed. The two lower curves show the corresponding behavior of the mutant.

The vertical scale on the right side shows ΔG for the light-driven step, calculated from Eqn. 3 with Eqns. 4–6, assuming that emission is entirely from PS II, that 63% of the absorbance at 680 nm is due to PS II, and that the action and emission spectra are as described in Marchiarullo and Ross, [21] and Melis et al. [29]. If the emission were entirely from PS I, then the ΔG scale would be shifted downward by 0.06 eV.

The two curves in Fig. 1 for a particular culture diverge at high light intensity because of the more rapid rate of decay of relative luminescence intensity under this condition. The two wild-type curves have a greater separation than the two mutant curves, showing that the wild-type has a larger rate of decay in the 1 s time region.

For simple enzyme kinetics, a log–log plot of velocity vs. substrate concentration will have a slope of 1/2 when half of the catalytic sites are occupied and the velocity is half of the maximum. For the 20–250 ms curve of the wild-type, the

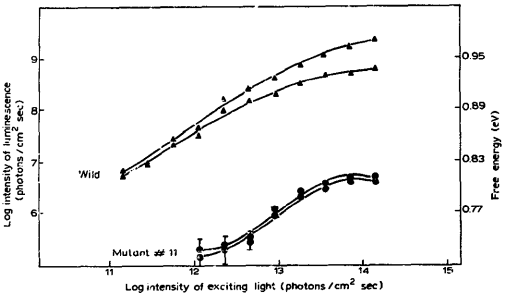

Fig. 1. Intensity of delayed luminescence from *Scenedesmus* as a function of exciting light intensity. Both axes are calibrated for absorption cross-section in dilute solution at 554 nm. Upper two curves, wild-type; lower two curves, mutant No. 11 deficient in plastoquinone A. Upper curve of each pair represents the average intensity 20 to 250 ms after illumination; lower curve, 1.5 to 2.5 s after illumination.

log–log plot of Fig. 1 has a slope of 1/2 at an excitation intensity of $8 \cdot 10^{13}$ photons $\cdot$ cm$^{-2} \cdot$ s^{-1}, which is equivalent to 0.3 W/m^2; this 'half-saturation intensity' will be used as a standard in further discussion. Both curves for the mutant have a slope of 0.5 at an intensity of $7 \cdot 10^{13}$ photons $\cdot$ cm^{-2} s^{-1}, and the 1.5–2.5 s curve for the wild-type has this slope at an intensity of $1 \cdot 10^{13}$ photons $\cdot$ cm$^{-2} \cdot$ s^{-1}.

At an excitation intensity of 0.3 W/m^2, the intensity of emission from the wild strain 10 ms after excitation corresponds to a free-energy change of 0.99 eV. The intensity of emission from the mutant is 310-times less than that from the wild strain. Were this emission due entirely to PS II, it would correspond to a ΔG of 0.83 eV; were it due entirely to PS I, it would correspond to a ΔG of 0.77 eV.

Fig. 2 compares the long-wavelength action spectra of mutant and wild samples. The difference shown is close to the experimental error.

Table I shows the relative emission in different wavelength bands from mutant and wild samples following excitation at different wavelengths. Our estimate of the emission from PS I is based on the assumptions (1) that all of the delayed emission

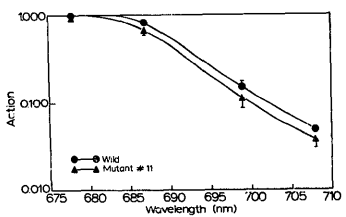

Fig. 2. Action spectra for wild and mutant No. 11. Action spectra normalized to 680 nm. The average for three different times from the decay curves (10 ms, 500 ms and 2.0 s) was used for each point in the graph.

TABLE I

RELATIVE EMISSION FROM WILD AND MUTANT NO. 11 WITH DIFFERENT WAVELENGTHS BANDS

	Fraction passed by cutoff filter		
	excitation (nm)	emission cutoff filter (nm)	
		695	715
Wild delayed	564	0.42	0.23
Wild prompt	564	0.51	0.27
Estimated PS I	564	0.60–0.72	0.31–0.36
Mutant delayed	564	0.50	0.32
	670	0.59	0.26
	688	0.46	0.27
	708	0.61	0.25

from the wild sample is from PS II and (2) that between 30 and 50% of the prompt emission from the wild sample is from PS I.

Finally, to test for the presence of more than one component in the luminescence from the mutant, the data used to compute the 'mutant-delayed' portion of Table I was placed in a three-way array: an array extending in three directions. This array was three excitation wavelengths (670, 688 and 708 nm) by three emission bands (emission longer than 650, 695 and 715 nm, respectively) by ten time delays since illumination (total luminescence measured in ten time-periods, each of equal width in log(time)). This array was subjected to three-mode principal component analysis [30]; this is a sensitive test for the presence of more than one spectral component, but only one was found.

From the results of the three-mode analysis, we estimate that emission with the spectrum characteristic of PS I comprises less than 20% of the total emission caused by excitation at any of the three wavelengths. Compared to wavelengths shorter than 680 nm, excitation at 708 nm favors PS I over PS II by a factor of 10 [31,32]. Combining these two figures, we estimate that the emission from PS I is less than 2% of the total emission from 10 ms to 2.5 s caused by excitation at wavelengths shorter than 680 nm. Very rapid decay kinetics would decrease the detectability of PS I emission, but not by more than a factor of 2 or 3. Thus we conclude that emission from PS I is less than 5% of the 10 ms emission at any wavelength.

If PS I did comprise 5% of this emission, its ΔG would be 0.77 eV + $k_B T$ ln (0.05), or 0.69 eV. This then becomes our provisional upper limit on the free energy generated by PS I.

Discussion

We measured the luminescence from wild-type and mutant No. 11 of *S. obliquus* under several conditions.

From the intensity of the luminescence from the wild-type, we compute the ΔG of PS II to be 0.99 eV at an excitation intensity of 0.3 W/m^2 at 564 nm, which is equivalent to 230 lux. This result is in agreement with our previous report [5]. This excitation intensity, identified earlier in this paper as that causing half-saturation of 20–250 ms luminescence, is also close to the compensation point, the light required for photosynthetic carbon fixation to equal carbon loss from respiration [33].

Our results with the mutant are in agreement with those of Bertsch et al. [8]: they found emission from the mutant to be 250-times weaker than emission from the wild-type between 0.75 and 4.2 ms after illumination with saturating light intensities. We found a ratio of 310 at 10 ms after saturating illumination.

We began this work with the hope that the emission from the mutant would be from PS I, so that the intensity of this emission would provide a direct measure of the ΔG of this photosystem. But what is the source of the emission? Several possibilities have occurred to us: (1) contaminant wild-type; (2) residual activity of mutated PS II; (3) a second kind of PS II; (4) PS I; or (5) some source other than the energy storage pathway of a photosystem. We note that the emission from the mutant and wild strains have different kinetics, which argues against the mutant emission being due to a contamination from the wild-type. On the other hand, the mutant and wild strains have similar excitation spectra, emission spectra, and saturation curves; we feel that this argues strongly against some some source other than a chlorophyll-driven photosystem.

This leaves us with a photosystem in the mutant as the source of luminescence. Fig. 2 shows the action spectrum of mutant luminescence to be, if different, at a wavelength shorter than that of the PS II responsible for luminescence from the wild strain ('normal PS II'); this is a good evidence that PS I is not the source, and suggests that 'normal PS II' may not be the source. On the other hand, Table I shows the emission of mutant luminescence to be at longer wavelengths than that of 'normal PS II', but with less emission beyond 715 nm than estimated for PS I.

In sum, we are certain that luminescence originates from Photosystem II. We are unsure whether this PS II is the one with plastoquinone A or another kind of PS II, such as the non-B type discussed in the current literature [7,34].

If the emission from the mutant had been due entirely to PS I, its intensity would have corresponded to a ΔG for that photosystem of 0.77 eV.

Based on an estimate that PS I is responsible for less than 5% of the emission from the mutant, we have calculated an upper limit of 0.69 eV for the free energy generated by this photosystem. This calculation is based on the questionable assumption of thermal equilibration within the excited state, but it represents the best available estimate of the efficiency of this light-energy conversion process. The reasons for the unexpectedly low efficiency remain unknown.

The free energies reported in this paper may appear to be inconsistent with the known properties of PS I and PS II. In particular, the primary donor of PS I has a midpoint potential of +0.45 V, and early acceptors have midpoint potentials between −0.5 and −0.7 V, suggesting a free-energy change of 1.0 V or more. This may seem to be in conflict with our finding that the actual free-energy change in PS I is 0.7 V or less.

The answer to this apparent conflict lies in the large difference between midpoint potentials and actual working potentials in efficient photochemical systems [35]. We recall that

$$E = E^{\circ\prime} - 0.059/n \log([\text{red}]/[\text{oxd}]) \quad (10)$$

where $E^{\circ\prime}$ is the standard electrode potential, [red] and [oxd] are the concentrations of the reduced and oxidized forms of the electron carrier, and n is the number of electrons transferred.

Most of the donor P-700 should be in the reduced state, ready for photochemistry. If the ratio [P-700]/[P-700$^+$] is between 100 and 1000 at light intensities just sufficient to drive net photosynthesis, then the corresponding potential of the primary donor is between +0.27 and +0.33 V. The secondary donor plastocyanin has a midpoint potential of +0.37 V [36].

The acceptor side of PS I has several very transient components which must be mostly in the oxidized state, ready to accept an electron from the excited P-700. Being highly oxidized at low light intensities, these components will have working potentials which are much less negative than their midpoints. These early acceptors are then followed by ferredoxin ($E^{\circ\prime} = -0.42$ V) and NADP ($E^{\circ\prime} = -0.33$ V). The NADP pool is 50% reduced in illuminated chloroplasts during CO_2 fixation [37].

With this reasoning, we suggest that PS-I under weak illumination operates between +0.3 and −0.4 V, for a free-energy change of 0.7 eV, in agreement with the results obtained from our luminescence measurements.

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