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LOW POTENTIAL TITRATION OF THE FLUORESCENCE YIELD CHANGES IN PHOTOSYNTHETIC BACTERIA

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SUMMARY

The fluorescence yield of whole cells of the photosynthetic bacteria *Chromatium*, *Rhodospirillum rubrum*, and *Rhodopseudomonas spheroides* was measured in low intensity light as a function of redox potential under anaerobic conditions at pH 8.0. The titration curves were fit by the Nernst equation for a one-electron reaction. The midpoint potentials were −160 mV and −145 mV, respectively, for *Chromatium* and *R. rubrum*. The *R. spheroides* showed less reversibility and is characterized by midpoints of −65 mV and −90 mV for the oxidative and reductive titrations.

INTRODUCTION

The redox potential of the primary electron acceptor in the photosynthetic bacteria has been estimated by titrating the light-induced absorbance changes of bacteriochlorophyll in *Rhodospirillum rubrum*[1] and cytochrome *c*-552 in *Chromatium*[2] as a function of redox potential. The decrease in the absorbance change at low potential was attributed to reduction of the primary acceptor for electrons transferred from reaction center bacteriochlorophyll. If the yield of fluorescence from the bulk bacteria chlorophyll depends on the redox state of the acceptor, as it does in the green plant system[3–5], this would provide an independent measure of the acceptor potential. It has been shown that there is a correlation between the fluorescence yield of photosynthetic bacteria and the oxidation state of the reaction center bacteriochlorophyll[6,7], with the midpoint potential of the reaction center of *R. rubrum* chromatophores being about +440 mV (ref. 8). We show here that there is a systematic and reversible variation in the fluorescence yield of the bacteria at much lower potentials, consistent with fluorescence quenching by the oxidized form of an acceptor.

EXPERIMENTAL PROCEDURE

Cultures of *Chromatium* strain D, *Rhodospirillum rubrum*, and *Rhodopseudomonas spheroides* were used in these experiments. Photoheterotrophic growth at 37° to stationary phase from 1:250 inocula required about 2 days under white light of intensity $9 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}. Heterotrophic medium for *Chromatium* was that

Abbreviation: E_{m7}, midpoint potential at pH 7.0.

used by CUSANOVICH AND KAMEN[9], and *R. rubrum* and *R. spheroides* were grown in a modified Hutner's medium. These cultures were washed twice and concentrated in phosphate buffer. Exponential phase cultures of the bacteria were obtained by growing for 16 h under the above conditions, which gave a cell density about 1/20 that of stationary phase. Broken cells in stationary or exponential phase were prepared in a French press at 1300 lb·inch^{-2}.

The fluorescence yield titrations were carried out at 25° in a medium consisting of 10 % sucrose, 0.1 M Tris–HCl buffer (pH 8.0) or 0.1 M Tris–HCl buffer (pH 8.0) and the following redox buffers, all at a concentration of 30 μM: anthraquinone 2-sulfonate ($E_{m_7} = -225$ mV); 2-hydroxy-1,4-naphthoquinone ($E_{m_7} = -145$ mV); 5-hydroxy-1,4-napthoquinone ($E_{m_7} = +30$ mV); and 1,4-naphthoquinone ($E_{m_7} = +60$ mV)[10]. The presence of these redox dyes allowed continuous buffered titration over a potential range of about -275 mV to $+100$ mV at pH 8.0. Anaerobic conditions were established as before[5] by bubbling through the redox cuvette AirCo high purity N_2 (< 10 ppm O_2) or Matheson prepurified argon (< 3 ppm O_2), with the O_2 content further reduced by passage through two wash bottles filled with chromous sulfate solution. The redox potential of the suspension was monitored by a platinum electrode inserted into the cuvette through a silicone rubber stopper. The accuracy of the electrode and the freedom of the suspension from contaminating O_2 was checked by titrating low potential dyes of known properties. Titrations of 30 μM FMN and neutral red at pH 7.0 gave very reversible two-electron titrations with midpoint potentials of -206 mV and -306 mV, respectively, using 186 mV, the calibration factor obtained in saturated quinhydrone solution, as the potential of the Ag–AgCl reference cell relative to the hydrogen couple.

Fluorescence was excited by chopped 585-nm monochromatic light of 55 ergs·cm^{-2}·sec^{-1} intensity and detected with a Dumont 6911 photomultiplier tube blocked by a Corning 7-69 color filter which has 70 % transmission at 900 nm. The modulated fluorescence signal was fed into a PAR lock-in amplifier and displayed on a strip-chart recorder as a function of time. Redox potential changes could be simultaneously read from a digital pH–millivolt meter (Instrumentation Lab. 205).

The titrations of fluorescence yield were generally started at low potential, about -275 mV, in order to further reduce the residual O_2 concentration and the oxidative titration was then done first. The titration procedure[5] consisted of adding a small amount (1–5 μl) of oxidant (0.1 M ferricyanide) or reductant (0.01 M dithionite in 0.01 M KOH) to the equilibrated suspension. Using whole cells of stationary phase bacteria suspended at 10–30 μM bacteriochlorophyll concentration in the redox medium described above, it takes 1–3 min for the system to reequilibrate at a new fluorescence level and at a new potential displaced from the initial value by 10–20 mV.

RESULTS

The fluorescence yield of suspensions of *Chromatium*, *R. rubrum*, and *R. spheroides* increases as the redox potential is lowered below 0 V under anaerobic conditions at pH 8.0 (Figs. 1–3). In contrast to the titrations of fluorescence yield for spinach chloroplasts[5] which show two components of approximately equal amplitude, there appears to be only one component in the titrations of the bacterial fluorescence. The oxidative and reductive titrations of the fluorescence from a whole cell suspension of

a stationary phase *Chromatium* culture can be fit fairly well by the Nernst equation for a one-electron transition with a midpoint potential of —160 mV (Fig. 1). The ratio of maximum to minimum fluorescence signal at the highest and lowest potential shown is 2.0:1. Very similar values for the midpoint potential have been obtained with broken cell preparations and subchromatophore particles from *Chromatium* (the latter experiment done in collaboration with Dr. M. CUSANOVICH), *Chromatium* grown photoheterotrophically as before, but at lower light intensities, and from *Chromatium* grown autotrophically with $NaHCO_3$ as the carbon source.

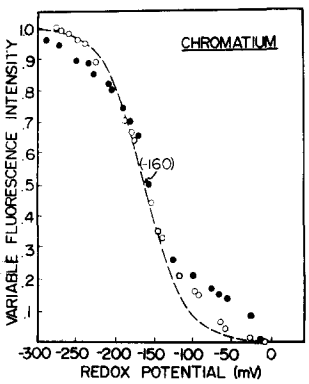
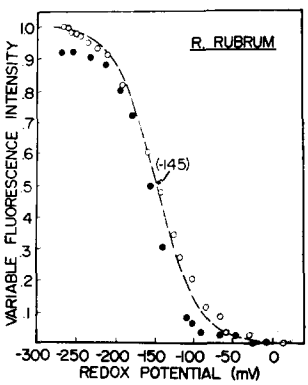


Fig. 1. Variation in fluorescence intensity of stationary phase cells of *Chromatium* as a function of redox potential at pH 8.0, at a bacteriochlorophyll concentration of 34 μM. The oxidative titration (○) is obtained by adding small aliquots (1–3 μl) of 0.1 M ferricyanide to the cell suspension initially at low potential. The reductive titration (●) employed 0.01 M dithionite. Measurements of fluorescence and potential were made after the system had reached equilibrium or an approximate equilibrium after each addition. The last ferricyanide addition made carried the potential to —6 mV, at which point the reductive titration began and, without any added reductant, proceeded slowly (elapsed time, 35 min) to —208 mV, when small aliquots of a dithionite solution were sequentially added to finish the reductive titration.

There is a very large amount of low potential endogenous reductant in the washed whole cell *Chromatium* preparations, enough to reduce the electrode potential below —200 mV at pH 8 without adding any other reductant. This endogenous reductant acts slowly enough so that it is possible to establish equilibrium over most of the potential range in the oxidative and reductive titrations. There is a discrepancy in Fig. 1 between the data points and the theoretical curve at potentials above —150 mV which we attribute to this endogenous reductant interacting rapidly enough with the fluorescence quencher that the latter is not able to equilibrate with an external oxidizing potential. At pH 7 and particularly at pH 6, the response of the fluorescence changes to variation of the potential is more sluggish and the deviation of the data from a theoretical one-electron curve at the high potential end of the titration is much more pronounced.

The Nernst equation for a one-electron transition with a midpoint potential of —145 mV provides a good fit to the oxidative titration of a suspension of stationary phase cells of *R. rubrum* (Fig. 2). The data points for the reductive titration are shown and they also fit the theoretical curve if they are normalized to 1.0. The ratio of the maximum fluorescence signal at low potential to the minimum fluorescence signal at high potential is 1.9:1 in this experiment.

The oxidative titration of *R. spheroides* has a midpoint potential of −65 mV and the data is fit best assuming a one-electron reaction (Fig. 3). The fluorescence was then titrated by reduction, with the maximum signal attained at low potential being 0.72 of the fluorescence at the start of the oxidative titration. The reductive titration is fit best by a curve for a one-electron reaction with an E_m of −90 mV. The ratio of maximum to minimum fluorescence yield in Fig. 3 is 2.1:1.



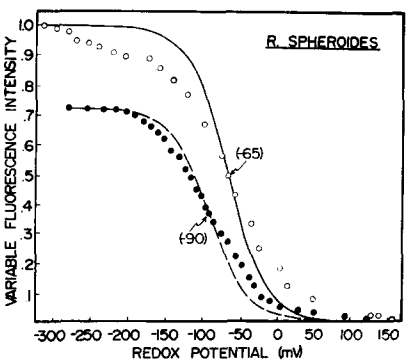


Fig. 2. Variation in fluorescence intensity of *R. rubrum* stationary phase cells at a bacteriochlorophyll concentration of 10 μM. Procedure as in Fig. 1 with an initial lowering of potential in the reductive titration solely due to endogenous reductant which decreases the potential to −109 mV in 34 min, when dithionite was added to finish the titration.

Fig. 3. Variation in the fluorescence intensity of *R. spheroides* stationary phase cells at a bacteriochlorophyll concentration of 10 μM. The endogenous reductant lowered the potential to −176 mV in 1 h and 15 min during the reductive titration.

Actinic light (595 nm, $5 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}) generally induces a 10–20% increase in signal above the maximum obtained at negative potentials in the anaerobic titrations. This may represent a fraction of the cell or chromatophore population which is relatively impermeable to reductant. The light-induced fluorescence yield changes obtained under aerobic conditions are between 2 and 3, as are the increases of fluorescence brought about by addition of dithionite powder in samples open to the air. Thus, the fluorescence yield changes being titrated here represent most of the change which can be seen.

It was not possible to systematically titrate the fluorescence yield of whole cells at positive potentials because they have only limited permeability to ferricyanide. Ferricyanide addition can cause a fluorescence yield increase in the region of +400–500 mV, presumably due to bleaching of the reaction center[7,11], but the response in whole cells is slow and the change in whole cells is never as large as that occurring at low potential. In broken cell preparations we can see small increases in the fluorescence yield at high potential, but these are often small compared to the decreases in fluorescence caused by ferricyanide addition. Such decreases in fluorescence have been seen by others[12]. We have found in *Chromatium* that the decreases in fluorescence are accompanied by extensive bleaching of the long wavelength bulk bacteriochlorophyll.

DISCUSSION

The midpoint potential of the *Chromatium* fluorescence yield changes (Fig. 1) is slightly more negative than that attributed to the acceptor of the light-induced cytochrome *c*-552 oxidation ($E_{m7.5} = -135$ mV)[2], and the latter appeared to be a two-electron change. The E_m of the fluorescence yield seen in reaction center particle preparations of *R. spheroides* is approximately -50 mV (ref. 13). The -145 mV midpoint of the *R. rubrum* titration curves measured in weak light at pH 8.0 and 25° (Fig. 2) is significantly more negative than the midpoint of the light-induced (-22 mV) and chemically-induced (-60 mV) bacteriochlorophyll absorbance changes determined by LOACH[1] at pH 7.6. A two-electron oxidation–reduction reaction seemed to fit most of the absorbance data, whereas the fluorescence yield titrations reported here are described by a one-electron function. A one-electron acceptor would be required if one reaction center molecule is the donor.

We have assumed in these experiments that the extent of fluorescence quenching is a measure of the state of oxidation of the primary acceptor. However, if there are one or more fast "dark" electron transfer steps down the electron transport chain which follow transfer to the acceptor, then what is measured here may not be the potential of the primary acceptor but that of a carrier further down the chain which precedes the first slow step. Without an inhibitor of electron transport blocking after the acceptor, or a mutant defective there, it is difficult to check this point.

The acceptor potentials inferred here from titrations of the fluorescence yield indicate that reduction of NAD^+ by the acceptor is not favored when the redox poise of the acceptor is near the midpoint. NAD^+ reduction by the acceptor can only become favored energetically if the acceptor is almost fully reduced. The magnitude of the light-induced fluorescence yield changes discussed above implies that in the light the acceptor is largely reduced or the reaction center oxidized. The extent of steady-state reduction required to lower the potential of the one-electron acceptors identified here is less than that required for a two-electron acceptor. For this reason we feel that direct photoreduction of NAD^+ cannot be ruled out solely by energetic considerations[1]. If the acceptor is poised 100:1 toward reduction by the light, then the acceptor potential would be -280 mV for *Chromatium* and -265 mV for *R. rubrum* at pH 8.0, which would be negative enough to allow appreciable NAD^+ reduction when NAD^+ is in excess. The lag seen[14] in the kinetics of reduction of NAD^+ in whole cells of *R. rubrum* and *R. spheroides* could then be ascribed to light energy required initially for the generation of a reduced steady-state of the acceptor. Independent of the energetic possibilities, however, is the biochemical evidence indicating that, at least in chromatophores of *R. rubrum*, NAD^+ is not photoreduced directly, but through energy linked reverse electron transport[15].

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REFERENCES

1 P. A. LOACH, *Biochemistry*, 5 (1966) 592.
2 M. A. CUSANOVICH, R. G. BARTSCH AND M. D. KAMEN, *Biochim. Biophys. Acta*, 153 (1968) 397.
3 L. N. M. DUYSENS AND H. E. SWEERS, *Studies on Microalgae and Photosynthetic Bacteria*, The University of Tokyo Press, Tokyo, 1963, p. 353.
4 W. L. BUTLER, *Biochim. Biophys. Acta*, 64 (1962) 309.
5 W. A. CRAMER AND W. L. BUTLER, *Biochim. Biophys. Acta*, 172 (1969) 503.
6 W. J. VREDENBERG AND L. N. M. DUYSENS, *Nature*, 197 (1963) 355.
7 R. K. CLAYTON, *Photochem. Photobiol.*, 5 (1966) 807.
8 P. A. LOACH, G. M. ANDROES, A. F. MAKSIM AND M. CALVIN, *Photochem. Photobiol.*, 2 (1963) 443.
9 M. A. CUSANOVICH AND M. D. KAMEN, *Biochim. Biophys. Acta*, 153 (1968) 376.
10 W. M. CLARK, *Oxidation–Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore, 1960.
11 R. K. CLAYTON, *Photochem. Photobiol.*, 5 (1966) 679.
12 B. C. MAYNE, *Biochim. Biophys. Acta*, 109 (1965) 59.
13 D. W. REED, K. L. ZANKEL AND R. K. CLAYTON, *Proc. Natl. Acad. Sci., U.S.*, 63 (1969) 42.
14 J. AMESZ, *Biochim. Biophys. Acta*, 66 (1963) 22.
15 D. L. KEISTER AND N. J. MINTON, *Biochemistry*, 8 (1969) 167.