Membrane-Bound *c*-Type Cytochromes in *Heliobacillus mobilis*. *In Vivo* Study of the Hemes Involved in Electron Donation to the Photosynthetic Reaction Center†

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ABSTRACT: The amount of heme per photosynthetic reaction center (RC) was examined in whole cells of *Heliobacillus mobilis*, and a stoichiometry of 5−6 hemes *c* and 1−3 hemes *b* per RC was found. Virtually the full complement of heme was seen to be functionally connected to the pool of electron donors to the photosynthetic RC. The kinetic parameters of electron transfer between reduced *c*-type hemes and the photooxidized primary donor P_{798}^+ were studied in whole cells and membrane fragments. The *in vivo* half-times of electron donation (50% with $t_{1/2} = 110$ μs, 50% with $t_{1/2} = 600$ μs) were seen to slow down to half-times in the range of several and several tens of milliseconds following disruption of cells. A severe conformational alteration or a change in the identity of the donating heme is discussed. Redox titrations of the flash-induced absorption changes performed on whole cells in the presence of mediators yielded the following redox midpoint potentials: P_{798}, $E_m = +240$ mV; heme c_{553}, $E_m = +190$, +170, and +90 mV for the heme components oxidized after the first, second, and third flash, respectively. The results demonstrate that the pool of c_{553} hemes donating electrons to the RC is heterogeneous and that it consists of either several distinguishable cytochromes or multiheme cytochromes or both. The number of hemes reduced and the kinetics of heme rereduction after flash-induced oxidation were found to depend strongly on the degree of anaerobicity in the interior compartment of the cell. A model rationalizing the obtained results in terms of a set of differing redox components is proposed.

Electron donation to the photooxidized pigment of the quinone- (RCII-) type photosystems from purple and green filamentous bacteria proceeds either directly from a soluble electron transfer protein or via an additional, RC-associated tetraheme subunit (Prince, 1990; Meyer & Donohue, 1995; Nitschke & Dracheva, 1995) interpositioned between the soluble carrier and the RC.[1] In purple bacteria, these processes were characterized in the 1960s [for a review, see Dutton and Prince (1978)]; however, the study of the underlying mechanisms and thermodynamics has regained considerable interest during recent years (Tiede et al., 1993; Ortega & Mathis, 1993; Venturoli et al., 1994) due to the presence of three-dimensional structures for all reaction partners involved.

Within the second group of reaction centers, i.e. the FeS- or RCI-type photosystems, the respective electron donation reaction has so far been characterized in significant detail only in PS1 from chloroplasts (Bottin & Mathis, 1987; Drepper, 1994; Diaz et al., 1994).

For the case of the remaining RCI-type photosystems found in green sulfur and heliobacteria (Pierson & Olson, 1989; Blankenship, 1992, 1994; Feiler & Hauska, 1995; Amesz, 1995), however, only few data have been reported with respect to these electron transfer reactions and some of the reported sets of data are controversial.

Heliobacteria, the RC of which has been shown to be a representative of the RCI family with respect to both structure (Liebl et al., 1993) and function [for a review, see Golbeck (1993)], were reported to contain a membrane-bound cytochrome c_{553}, acting as the reductant of the photooxidized primary donor P_{798}^+ in membrane fragments with half-times in the range of 6−20 ms (Fuller et al., 1985; Prince et al., 1985; Kleinherenbrink & Amesz, 1993). In whole cells, however, flash-induced oxidation of heme with an α-band maximum at 553 nm has been shown to occur significantly faster (100−700 μs, Vos et al., 1989). On the basis of the results obtained on whole cells, electron transfer was proposed to proceed linearly from reducing substrates (e.g. ascorbate) through the RC to NADH. Subsequently, however, the presence of a cytochrome *bc* complex has been demonstrated (Liebl et al., 1990), and it was shown that, in whole cells, the c_{553} hemes mediate electron flow from the cytochrome *bc* complex toward the RC (D. M. Kramer, B. Schoepp, B. Floss, U. Liebl, and W. Nitschke, unpublished results). In apparent conflict with such a function, a photoinduced reduction of cytochrome c_{553} without involvement of the cytochrome *bc* complex was reported by

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[1] Abbreviations: PMS, phenazine methosulfate; PS1, photosystem 1; RC, reaction center.

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Kleinherenbrink and Amesz (1993) in membrane fragments under oxidizing conditions.

The number of cytochrome c_{553} hemes per RC was reported to be in the range of 1−2 (Kleinherenbrink & Amesz, 1993; Vos et al., 1989). This stoichiometry would be in disagreement with the proposal that cyt c_{553} might be a tetraheme cytochrome related to the respective proteins in purple bacteria and *Chloroflexus* (Nitschke & Rutherford, 1991) but in line with the hypothesis of cytochrome c_{553} being a monoheme subunit (Okkels et al., 1991) in analogy to a similar hypothesis put forward for green sulfur bacteria.

In order to assay the validity of the hypotheses presented so far, we studied the *c*-type heme components both in whole cells and in membrane fragments from *Heliobacillus mobilis* with respect to their spectral, kinetic, and electrochemical properties. The various models proposed so far are discussed on the basis of these data.

EXPERIMENTAL PROCEDURES

Cultures of *H. mobilis* (kindly provided by Dr. R. E. Blankenship, Tempe, AZ) were grown as described by Liebl et al. (1990). Using 0.5% inocula, the cultures were found to achieve late logarithmic growth phase after about 1.5 days. At 2 days, the stationary growth phase was reached, accompanied by the appearance of high amounts of lysed cells and rapid loss of photoinduced absorbance changes (in 2.5-day-old cultures, only 10% of the maximal signals observable after 1.5 days remained). All experiments on whole cells described in this work were therefore performed on cultures 1.0−1.5 days of age. In this time range, cultures showed an average OD_{780} of 0.5 in the measuring cuvette (path length of 1.6 cm). Aliquots were transferred via air-tight syringes from the rubber-stoppered culture bottles into the argon-flushed cuvette compartment. For specific experiments (see Results), cells were spun down (GSA rotor at 5000 rpm for 10 min) and resuspended in degassed Tris- or MOPS-buffer (50 mM, pH 8.0 or 7.0, respectively) to the same OD as measured in the growth medium before centrifugation.

Sphaeroplasts and membrane fragments were prepared by adding lysozyme (100 nM final concentration) to the measuring cuvette containing suspensions of whole cells. Details of the time course of cell lysis are given in the Results.

Redox poising and titrations in whole cells were performed essentially as described by Dutton (1971) using the following mediators: 1,4-benzoquinone, 2,3,4,5-tetramethyl-*p*-phenylenediamine, 2,5-dimethyl-*p*-benzoquinone, 1,2-naphthoquinone, phenazine methosulfate, 5-hydroxy-1,4-naphthoquinone, duroquinone, 2,5-dihydroxy-*p*-benzoquinone, and 2-hydroxy-1,4-naphthoquinone at 500 nM. Additions of chemical oxidants/reductants induced changes in the redox states of P_{798} and hemes. Their respective degree of reduction or oxidation was monitored in the spectral region of 400−600 nm. Equilibration was considered to be complete when no more spectral changes could be observed. This process usually took 1−5 min, depending on the extent of the redox jump and the value of the ambient redox potential at the end of the jump. Significantly higher concentrations of mediators resulted in uncoupling of electron transfer between the observed redox components. Even at a concentration of 500 nM, the mediators were found to interfere with transport of the positive charge toward tertiary electron donors such as the cytochrome *bc* complex and the menaquinone pool. It is of note that the addition of mediators resulted in samples yielding reproducible kinetic results for several hours. However, these cell suspensions were not competent for further inoculation.

Spectrophotometric measurements were performed in an apparatus similar to that described by Joliot and Joliot (1984). Actinic excitation was provided by the following light sources: a ruby laser (694 nm, 50 ns), a dye-laser (oxazyme 720, 697 nm, 500 ns), and a Xenon flashlamp (Hamamatsu L2445, 3 μs duration at half-height). In all experiments using only one actinic flash, this light was provided from either the ruby or the dye laser. In the experiments described in Figures 3 and 4, however, the dye laser provided the first flash whereas the second and the third flashes were provided by the ruby laser and the Xenon flashlamp, respectively. In the experimental setup permitting a combination of flashes from different light sources, the Xenon flashlamp was found to be only 60% saturating (due to losses in the optics used to merge the three different actinic beams). In the multiple flash experiments shown in Figure 6, the merging optics were omitted, resulting in saturating illumination also from the Xenon flashlamp.

Continuous illumination was provided from two projector lamps filtered through a 720 nm cutoff filter, resulting in a maximal light intensity of 200 μmol of photons m^{-2} s^{-1} incident on the sample.

RESULTS

Determination of Cytochrome Content in Whole Cells of H. mobilis. The full complement of cytochromes in *H. mobilis* was assayed by recording chemical difference spectra in whole cells. Addition of 2 μM PMS resulted in equilibration (<10 s) of all observable redox components with ambient redox potentials imposed by ferricyanide, ferrocyanide, ascorbate, or dithionite added to the cell suspensions. Figure 1a shows that (with respect to the oxidized state in the presence of a 10 mM stoichiometric mixture of ferri- and ferrocyanide) the addition of ascorbate resulted in the reduction of *c*-type hemes with α-, β-, and γ-bands at 553.5, 525, and 422 nm, respectively. The ascorbate-reducible heme made up for about 50% of the full content of *c*-type heme as measured after the subsequent addition of dithionite (Figure 1a). In addition, *b*-type hemes were reduced by dithionite to an extent of roughly 30% of the total amount of heme *c* (as evidenced by the pronounced shoulder at 562 nm and the shift of the γ-peak toward 427 nm).

Cytochromes Functionally Connected to the Photosynthetic RC. The amount of photooxidizable heme was measured in cell suspensions in the absence of chemical reductants/oxidants and PMS. When air was bubbled through such a cell suspension for 5−10 s (in the absence of PMS and chemical oxidant/reductant), all cytochromes became oxidized. In this state, even P_{798} was in the oxidized state as evidenced by the absence of a light-induced bleaching of the primary donor and the appearance of characteristic negative bands at 575 and 592 nm (see below). However, during subsequent dark incubation, metabolic processes decreased the ambient redox potential of the intracellular compartments, thereby reducing first P_{798}^{+} and subsequently

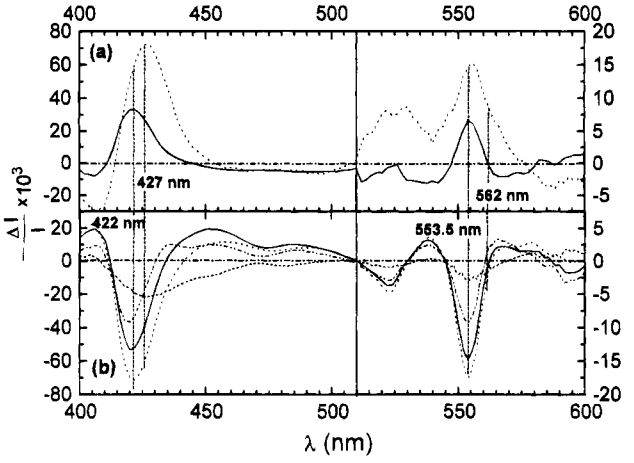

FIGURE 1: (a) Absorption difference spectra recorded on whole cells poised to three different redox conditions by addition of an equimolar mixture of ferri- and ferrocyanide (5 mM each, "oxidized state"), ascorbate (5 mM, "moderately reduced state"), and dithionite (5 mM, "fully reduced state") in the presence of 2 μM PMS. The continuous line represents the difference between the moderately reduced and the oxidized states, whereas the dotted line shows the difference between the fully reduced and the oxidized states. (b) Absorption changes in intact cells and sphaeroplasts induced by continuous illumination with saturating far-red light. The continuous and dotted lines represent difference spectra obtained on cells in the semianaerobic and anaerobic states (see text), respectively. The dashed spectrum is the difference between the spectra drawn with continuous and dotted lines. The dash-dotted spectrum was recorded on sphaeroplasts as described in the text. The right and left y-axis scales refer to spectral regions below and above 510 nm, respectively.

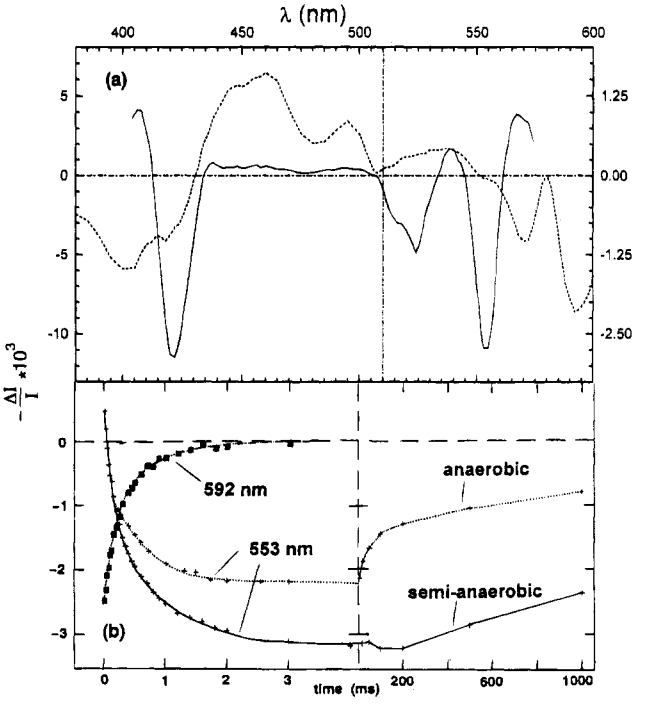

FIGURE 2: (a) Flash-induced absorption difference spectra of P_{798}^+/P_{798} as measured 10 μs after the actinic flash (dashed curve) and heme c_{553}^{ox} heme c_{553}^{red} taken 100 ms after the flash (continuous curve) recorded on whole cells of *H. mobilis* in the semianaerobic state. (b) Kinetics of photooxidation of heme c_{553} and rereduction of P_{798}^+ measured at 553 nm (crosses) and at 592 nm (squares) in the semianaerobic (continuous line) and the anaerobic (dotted line) state of the cell suspension. Kinetics at 592 nm (broken line, squares) were found to be identical in the anaerobic and the semiaerobic state.

the cytochrome complement. After 2−5 min of incubation in darkness, illumination with constant saturating far-red light (>700 nm) induced the difference spectrum shown in Figure 1b (continuous line). This spectrum is dominated by the continuous wave (cw) light-induced oxidation of *c*-type hemes. This respective redox state of the cells will in the following be referred to as the "semianaerobic" state. Prolonged incubation of the sample in darkness (>15 min) and subsequent illumination by cw light resulted in a difference spectrum characterized by an increased (by a factor of 1.2) oxidation of *c*-type hemes (Figure 1b, dotted line). In addition, the oxidation of *b*-type hemes became visible in the region around 560 nm (i.e. in the vicinity of the isobestic point of c_{553} hemes) and in the region of the γ-bands (see especially the dashed difference spectrum in Figure 1b). Since the *b*-type hemes in *H. mobilis* titrate between −100 and −200 mV (D. M. Kramer, B. Schoepp, B. Floss, U. Liebl, and N. Nitschke, unpublished results), the ambient redox potential in the environment of these hemes in extensively dark adapted living cells must be close to or lower than −200 mV. This state will henceforth be referred to as the "anaerobic" state (for a more instrumental definition of these states, see the section on Kinetics of Electron Donation Reactions under Various Redox Conditions).

A comparison of parts a and b of Figure 1 shows that more than 80% of the total chemically reducible hemes (Figure 1a) could be oxidized by saturating cw light, i.e. the majority of hemes was ultimately able tc denote their electrons into the photodriven electron transfer chain. The relative fraction of photooxidized *b* hemes was significantly lower than that of photooxidized c-type hemes. The photoinduced changes in the reduction state of *b*-type hemes were studied in detail (D. M. Kramer, B. Schoepp, B. Floss, U. Liebl, and N. Nitschke, unpublished results) and will be reported elsewhere.

A different result was found when cells were incubated with lysozyme prior to the experiments (see the section on Alterations of Cytochrome Photooxidation Reactions Induced Disruption of Cells). Under these conditions, the oxidation of about half of the full complement of *c*-type hemes and essentially no redox changes in cytochrome *b* were induced by continuous illumination (Figure 1b, dash-dotted line). Addition of 500 μM dithionite at pH 8.0 did not increase the amount of photooxidizable heme (not shown), demonstrating that the lower amount of photooxidizable heme was not merely due to a shift in the ambient redox potential preoxidizing a fraction of hemes prior to the illumination.

Kinetics of Electron Donation Reactions under Various Redox Conditions. When a single turnover saturating laser flash was given to a suspension of cells in the semianaerobic state, i.e. either in between 1 and 10 min after aeration (P_{798}^+ was found to be fully reduced within a few seconds of darkness after aeration) or poised to redox potentials of around +100 mV (see the section on Redox Dependence of the Oxidation and Rereduction Phases), the appearance of the characteristic spectrum of the photooxidized primary donor P_{798}^+ was observed shortly after the flash [at 10 μs, Figure 2a, dashed curve; cf. Prince et al. (1986)]. P_{798}^+ was subsequently rereduced with biphasic kinetics (as measured at 592 nm, Figure 2b). With matching kinetics, a *c*-type heme with an α-band at 553 nm became oxidized (Figure 2a, continuous curve). The kinetics both at 553 and at 592 nm (Figure 2b) could be fitted by two exponential curves of

equal amplitudes and half-times of 110 and 600 μs, respectively. It is of note that the extent of heme oxidized by a single turnover flash amounted to only about one-sixth of the full complement of *c*-type heme as determined from Figure 1 (see below).

The matching kinetics suggest that one heme c_{553} is oxidized per P_{798}^+ reduced. Assuming an extinction coefficient (ϵ) of 20 mM^{-1} cm^{-1} for the α-band of *c*-type hemes, this stoichiometry would result in an extinction coefficient of 16 mM^{-1} cm^{-1} for P_{798}^+ at 592 nm (compare Figure 2a, continuous curve at 553 nm vs dashed curve at 592 nm). In the specific experiment of Figure 2, red actinic flashes were used, precluding measurements in the region of the Q_y band of P_{798}. However, in independent experiments (N. Nitschke and P. Mathis, unpublished results), the extinction coefficients for the P_{798}^+/P_{798} difference spectrum were determined in the region between 380 and 1500 nm (to be published elsewhere). These data yield ϵ-values of 16 and 240 mM^{-1} cm^{-1} at 592 and 798 nm, respectively. The agreement both between these data and the ϵ-value deduced from Figure 2a and between our value and that reported by Kleinherenbrink et al. (1994) at 798 nm demonstrates that indeed one heme c_{553} is photooxidized per P_{798}^+.

Under these redox conditions, photooxidized heme c_{553} remained largely oxidized on the time scale of the experiment (see Figure 2b, continuous curve) and its full rereduction took several seconds, depending on the degree of aeration prior to the flash. In contrast, when cells were allowed to achieve anaerobicity (i.e. after >15 min of dark incubation in the absence of oxygen), the flash-induced photooxidation of heme c_{553} reversed within several hundreds of ms (Figure 2b, dotted curve). This rereduction phase of heme c_{553} was biphasic; both phases became more rapid and increased in relative amount with increasing anaerobicity inside the cells (cf. Figure 5). The redox potential dependence of the rereduction event is further described below (see the section on Redox Dependence of the Oxidation and Rereduction Phases).

The maximal extent of the cytochrome oxidation signal at 553 nm observed after a single, saturating flash was significantly decreased in anaerobic cells (to about 70% of the extent observed in the semiaerobic state, see Figure 2b). However, since the kinetics of P_{798}^+ rereduction measured under anaerobic and semiaerobic conditions were found to be superimposable, the apparently reduced extent of oxidized heme c_{553} and the speeding up of the slower half of the oxidation kinetics must be a consequence of a competition between the slower phase of oxidation by P_{798}^+ and the relatively rapid phases of rereduction from further electron donors (e.g. the turnover of the *bc* complex, see below). In fact, kinetic simulations using the experimentally determined rate constants for the oxidation and reduction kinetics agree well with the measured decrease in observable cytochrome c_{553} oxidation as already reported by Vos et al. (1989).

It has been claimed previously (Vos et al., 1989) that cells transferred from (ascorbate-containing) growth medium into reductant-free Tris-buffer no longer showed the cytochrome c_{553} rereduction phase in the millisecond range. However, we found that the reason for this effect was that, after such a medium exchange, the reestablishment of anaerobic conditions took significantly longer (up to 45 min) than after short aeration. Indeed, after anaerobicity has been reestablished, the observed kinetics are indistinguishable from those

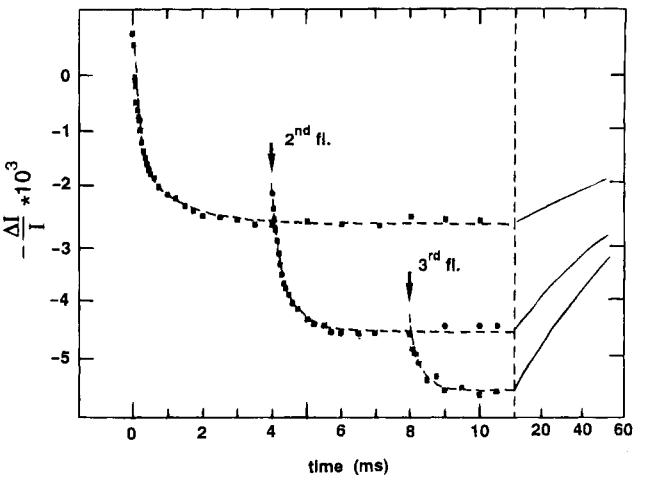

FIGURE 3: Kinetics of photooxidation of c_{553} hemes following one, two, or three consecutive actinic flashes spaced by 4 ms intervals (for details, see Experimental Procedures). The broken lines represent fits to the data points assuming the following half-times: biphasic oxidation with 50% at 110 μs and 50% at 600 μs; multiphasic rereduction kinetics with largely dominating contributions of 110 ms (only one flash), 80 ms (after two flashes), and 50 ms (after three flashes) half-times.

described above. We therefore conclude that, apart from a more thorough aeration of the cells, there is no difference in the light-induced electron transfer between cells in reductant-containing growth medium and in reductant-free isotonic buffer.

In the experiments reported previously [cf. Figure 7 in Vos et al. (1989)], only 1.7 positive charges could be extracted from the heme system by multiple flashes. This is in striking contrast to our observations. When multiple saturating flashes were given to a sample in the anaerobic state, significantly more than 2 hemes could be photooxidized. As shown in Figure 3, three flashes given at 4 ms intervals (i.e. leaving sufficient time for complete oxidation of cytochrome c_{553} by the RC between flashes) resulted in the oxidation of roughly 3 hemes per P_{798}^+ (note that the third flash came from a Xenon flashlamp which was determined to be only 60% saturating in independent experiments). It is of note that the kinetics of the second and third oxidations were characterized by the same set of biphasic kinetic parameters as determined for the oxidation following the first flash if the increased rates of the rereduction kinetics were taken into account (see above). In a train of flashes given to cell suspensions in the fully anaerobic state, a maximal amount of 5−6 hemes c_{553} per P_{798} could be photooxidized (see Figure 6).

The amount of heme c_{553} oxidizable by a train of flashes decreased with decreasing anaerobicity (induced by injecting microliter amounts of air into the sample), approaching about 2 heme per P_{798}. Higher oxygen tensions decreased the oxidation of heme after the first and second flash in almost equal proportions (see the section on Redox Dependence of the Oxidation and Rereduction Phases). We therefore conclude that the data of Vos et al. (1989), showing a total of 1.7 hemes photooxidized in a train of flashes, can be explained by assuming that the samples were not entirely anaerobic, thereby masking the actual maximal amount of photooxidizable heme.

Redox Dependence of the Oxidation and Rereduction Phases. The presence of redox mediators at low concentra-

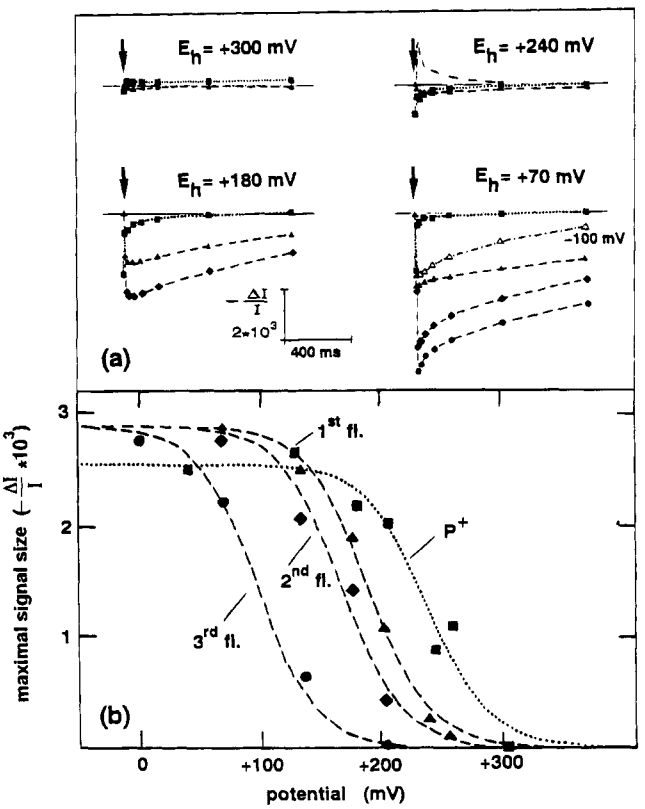

FIGURE 4: Redox potential dependence of the kinetics measured at 553 and 592 nm. (a) Kinetics observed at E_h values of +300, +240, +180, and +70 mV induced by one, two, or three actinic single turnover flashes spaced by 4 ms (for details, see Experimental Procedures). The dotted lines (squares) represent absorption changes due to P_{798}^+ (measured at 592 nm), whereas heme c_{553} changes (at 553 nm) are indicated by dashed curves triangles, diamonds, and circles denoting first, second, and third flash-induced oxidations). The dash-dotted upward kinetics at +240 mV represents photoinduced reduction of c_{553} heme seen in membrane fractions obtained by sonication. (b) Redox titration of the absorption changes indicated in part a. Data points obtained at 592 nm (P_{798}^+) are marked by squares, and those measured at 553 nm following one, two, or three actinic flashes are represented by triangles, diamonds, and circles, respectively. Data points obtained for heme c_{553} oxidation after the second and third flash were normalized to maximal values identical to those measured after the first flash. Curves through the data points (broken lines for data obtained at 553 nm and dotted lines for those measured at 592 nm) are theoretical Nernst curves, assuming $n = 1$ behavior and E_m values of +240 mV (592 nm), +190 mV (553 nm, first flash), +170 mV (553 nm, second flash), and +90 mV (553 nm, third flash).

tions (500 nM, see Experimental Procedures) in the suspensions induced slow equilibration of the spectroscopically observed redox components (P_{798} and hemes) with the externally applied ambient redox potentials. After equilibration had been achieved, the electron transfer reactions were triggered by one, two, or three actinic flashes. The respective kinetics recorded at 592 nm (to monitor P_{798}^+, squares, dotted lines) and at 553 nm (observing redox reactions of hemes), at a range of redox potentials, are shown in Figure 4a. At $E_h > +300$ mV, essentially no flash-induced changes of P_{798} and heme c_{553} occurred. Below +280 mV, photooxidation of P_{798} was observed. In the range of +280 to +240 mV, rereduction of P_{798}^+ was found to take several hundred milliseconds. This, together with the absence of significant absorption changes at 553 nm (neither oxidation nor reduction, see below), suggested that the physiological electron donor to P_{798}^+, i.e. a c_{553} heme, was still in the oxidized state. We assume that the donors to P_{798} on the hundreds of milliseconds time scale were the added mediators. Figure 4b shows the full redox titration curve of P_{798} photooxidation measured at 593 nm 10 μs after the flash. The data points could be fitted with an $n = 1$ Nernst curve, assuming a midpoint potential of +240 mV.

Below 200 mV, both the appearance of a fraction of rapidly (<1 ms) rereduced P_{798}^+ and simultaneous oxidation of heme at 553 nm became visible (Figure 4a). The extent of single flash-induced absorbance changes at 553 nm titrated with an apparent E_m of +190 mV ($n = 1$), as depicted in Figure 4b. When a second laser flash was applied to the sample 4 ms after the first, a further (but smaller) oxidation was observed. It is of note that this effect could also be observed at ambient redox potentials where less than 1 heme c_{553} per P_{798} was reduced prior to the actinic flash so that a single actinic flash oxidized significantly less than 1 heme per P_{798} (see Figure 4a, traces taken at $E_h = +180$ mV). In other words, even though an excess of P_{798}^+ was available, not all heme c_{553} could be oxidized, yet additional oxidation could occur after the second.

In the region of ambient potentials where the heme oxidation following the first flash titrated in, the amount of heme oxidized after the second flash was always lower than the extent of oxidation induced by the first flash. Figure 4b shows that the E_m values of the respective titration curves for the first and second flash oxidation differ by about 20 mV.

Below E_h values of about +160 mV, heme c_{553} oxidation was also observed following the third flash (Figure 4a). This third flash photooxidized heme titrated with an E_m value of about +90 mV (Figure 4b).

In the presence of mediators, the kinetics of heme c_{553} rereduction were modified as compared to those observed with untreated cells. At high redox potentials, corresponding to semiaerobic conditions, i.e. where in untreated cells rereduction took several seconds (see Figure 2b), a rereduction phase in the range of several hundred milliseconds was observed. In contrast, whereas the rereduction phases in untreated cells were accelerated by more than 100-fold on going from semiaerobic to anaerobic conditions (about 10 s to 50 ms), the corresponding acceleration of this phase did not exceed a factor of 20 in mediator-treated samples. This attenuation was most likely due to competing oxidation/reduction reactions of the mediator cocktail with components of the secondary electron transport chain on the time scale of hundreds of milliseconds.

This decrease in the extent of acceleration rendered an exact determination of the midpoint potential of this effect difficult. Nevertheless, from the data, it can be estimated that the appearance of the rereduction phase titrates between +100 and −100 mV.

It is noteworthy that, at E_h values above +200 mV, the kinetics of heme c_{553} oxidation deviated from the typical biphasic behavior. Detailed kinetics, however, are difficult to record due to the small extent of heme oxidation at these potentials. Furthermore, since P_{798} is also titrating within this range of ambient potentials, a complicated situation of mixed oxidation states in P_{798} and heme c_{553} must be taken into consideration.

It is furthermore of note that, in whole cells at moderately high potentials, i.e. when P_{798} was reduced but no oxidation

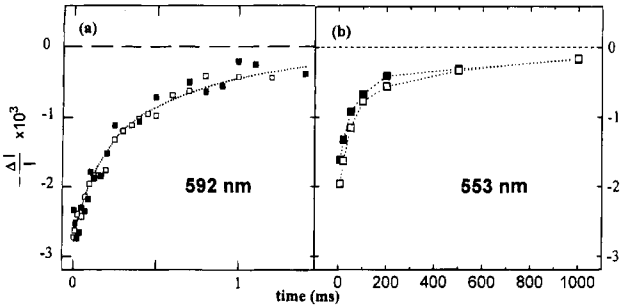

FIGURE 5: Comparison of flsah-induced absorption changes at 592 nm (a) and at 553 nm (b) using 100% (open squares) and 10% (filled squares) saturating flashes. The absorption changes following excitation of only 10% of the centers were normalized to those obtained using saturating flashes 100 μs after the flash. Part a shows kinetics measured at 592 nm, representing rereduction of P_{798}^+ by heme c_{553}, whereas part b depicts rereduction kinetics of heme c_{553} by secondary donors on a longer time scale.

of heme c_{553} could be detected, there was no measurable reduction of heme c_{553} either, in contrast to the reduction kinetics observed on membrane fragments by Kleinherenbrink and Amesz (1993). However, when the cell suspension was sonicated for 2 min to disintegrate the cell compartment, a reduction phase appeared (Figure 4a, dashed upward curve at +240 mV). This kinetic component was reproducibly found only when mediators were present. Sonication of untreated cells and poising at the required ambient potential with ferricyanide resulted in significant variations of this phase between different experiments (see Discussion).

Effects of Subsaturating Excitation on the Observed Electron Transfer Events. As described in the section on Kinetics of Electron Donation Reactions under Various Redox Conditions, a saturating single turnover flash applied to cells in the fully anaerobic state induced electron donation from heme c_{553} to P_{798}^+ with biphasic kinetics in the submillisecond range and a subsequent rereduction of the cytochrome system lasting a couple of hundreds of milliseconds. A decrease of the flash intensity down to about 10% saturation, i.e. producing only 10% of photooxidized P_{798}^+ (measured at 592 nm), as compared to the signal seen after saturating flashes yielded kinetics as shown in Figure 5. P_{798}^+ rereduction by the hemes was unchanged within the accuracy of the measurement (Figure 5a). The electron donation from heme c_{553} to P_{798}^+ therefore shows the characteristics of a first-order process.

The rereduction of heme c_{553}, however, was slightly accelerated (Figure 5b). Concomitantly, the extent of the bleaching at 553 nm (see first data point at 7 ms) decreased due to more efficient competition between the rereduction (accelerated) events and the oxidation (invariant) kinetics (note that, in Figure 5b, kinetics at 100% and at 10% saturation were normalized to equal amplitudes at 100 μs after the flash).

Alterations of Cytochrome Photooxidation Reactions Induced by Disruption of Cells. With the exception of the work by Vos et al. (1989) on whole cells, all previous studies on cytochrome oxidation by P_{798}^+ in heliobacteria have been performed on membrane fragments (Prince et al., 1985; Smit et al., 1987; Kleinherenbrink & Amesz, 1993). Since significant discrepancies exist between the results obtained on membrane fragments and whole cells, we repeated some of these experiments on membrane fragments also.

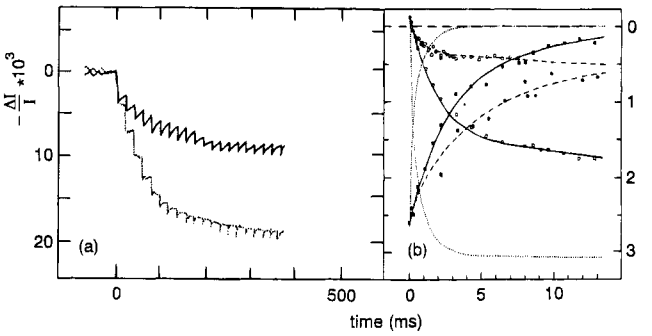

FIGURE 6: (a) Absorption changes at 553 nm induced by a train of saturating Xenon flashes separated by 20 ms intervals. The dotted curve represents kinetics measured on whole cells, and the continuous line represents the respective kinetic trace observed on the same sample after 1 h of incubation with lysozyme. (b) Kinetics of cytochrome oxidation (measured at 553 nm, open symbols) and of P_{798}^+ (measured at 592 nm, filled symbols) in whole cells (dotted lines), after 1 h (squares, solid lines) and after 4 h (circles, broken lines) of lysozyme treatment. Actinic excitation was provided by single turnover saturating flashes from the ruby laser.

In the previous studies cited above, membrane fragments were produced by either sonication or French Press treatment. For the experiments described below, we decided to use the "milder" lysozyme treatment. Addition of lysozyme to cell suspensions resulted (within about 5 min) in loss of ability to induce anaerobic reducing conditions in the cell interior. Prolonged incubation (>15 min) first induced the formation of sphaeroplasts that subsequently tended to lyse, resulting in a suspension of membrane fragments within a few hours after addition of lysozyme. After 6 h of lysozyme treatment, samples strongly resembled those after sonication or French Press treatment. However, disruption of cells by lysozyme offers the advantage that this treatment can be performed within the cuvette, allowing continuous monitoring of alterations in electron transfer during cell rupture.

Figure 6a shows absorption changes measured at 553 nm induced by a train of saturating flashes separated by 20 ms intervals prior to and after 1 h of lysozyme treatment. In intact cells in the anaerobic state, the first four flashes induced oxidations of 1, 0.94, 0.82, and 0.82 hemes c_{553} per P_{798}^+, respectively. After the fifth flash, 0.50 heme per P_{798}^+ was oxidized, and after further flashes, the stoichiometry dropped drastically. In total, about 5−6 hemes per P_{798}^+ were oxidized when saturation had been reached. In membrane fragments, the amount of heme oxidized by P_{798}^+ in the first five flashes was slightly but measurably diminished compared to that of the intact cells (0.94, 0.75, 0.60, 0.60, and 0.50 hemes per P_{798}^+ for the first five flashes, respectively). The decreased efficiency of cytochrome oxidation was paralleled by the appearance of a fraction of P_{798}^+ which became rereduced very slowly (see below). Furthermore, the maximal amount of photooxidizable heme decreased to about 2.5 hemes per P_{798}^+. Addition of dithionite (100 μM) increased this stoichiometry to about 3 hemes per P_{798}^+. Thus, the lysozyme treatment functionally disconnected a part of the cytochrome pool from the RC.

The above-described kinetics remained constant for several hours. About 4 h after addition of lysozyme, however, a further decrease in the total amount of photooxidized heme c_{553} became apparent, yielding samples with significantly less than 1 heme per P_{798}^+ oxidized by a train of flashes. More prolonged incubation resulted in a progressive loss of flash-

induced bleaching at 592 nm, indicating damage of the RC itself.

These data suggest that, during formation of membrane fragments, hemes c_{553} became increasingly functionally detached from the RC. An inspection of the time-resolved electron donation from heme c_{553} to P_{798}^+ during this treatment corroborates this hypothesis.

Figure 6b shows a comparison of kinetics mesaured at 592 and 553 nm during different stages of formation of membrane fragments. Whereas in intact cells the respective kinetics were found to match (taking into account the distortion of heme c_{553} oxidation at times longer than 500 μs due to the rapid rereduction processes), an increasing discrepancy between the respective redox changes was induced by the lysozyme treatment. After cell lysis was accomplished, the sample became heterogeneous, with only a fraction retaining flash-induced cytochrome oxidation, albeit with significantly slower rates ($t_{1/2}$ of 1.4 ms and several tens of milliseconds; Figure 6b, squares, continuous lines). In the remaining fraction, heme c_{553} could no longer donate electrons to P_{798}^+. In these centers, P_{798}^+ was rereduced with $t_{1/2} = 6$ ms, most probably due to back-reaction from reduced acceptors [corresponding to the $\tau = 8$ ms phase described by Kleinherenbrink and Amesz (1993)]. Prolonged incubation with lysozyme (several hours) resulted in a steady increase of the amount of P_{798}^+ functionally disconnected from the cytochrome system (Figure 6b, circles, broken lines). ' For comparison, the *in vivo* kinetics measured at 592 and 553 nm are indicated by dotted lines in Figure 6b.

Treatment of heliobacterial cells which are known to be devoid of a lipopolysaccharide-containing outer layer (Beck et al., 1990) with lysozyme thus severly perturbs the structural integrity of the c_{553} hemes with respect to the RC.

DISCUSSION

The Donation Reaction. In whole cells of *H. mobilis*, photooxidized P_{798}^+ is rereduced by a heme characterized by an α-band at 553 nm. In redox titrations of the flash-induced electron transfer events from heme c_{553} to P_{798}^+ in whole cells in the presence of membrane-permeable redox mediators, the redox midpoint potentials of P_{798} and the donating heme c_{553} were determined at +240 and +190 mV, respectively. The difference spectra of P_{798}^+/P_{798} and of heme c_{553} corresponded well to the respective spectra measured previously on membrane fragments of *H. chlorum* (Prince et al., 1985). The E_m values determined in our study for *H. mobilis* are higher by roughly 20 mV with respect to those reported by Prince et al. (1985) for membrane fragments of *H. chlorum*. Since the Rieske 2Fe2S cluster in *H. mobilis* was also found to have a measurably higher E_m value compared to its counterpart in *H. chlorum* (Liebl et al., 1990; Liebl, 1993), we tend to consider the above-mentioned higher E_m values as being due to species variability rather than arising from differences in sample intactness.

It is of note that these E_m values result in a driving force for electron donation from heme c_{553} to P_{798}^+ that amounts to only about 50 mV, i.e. significantly less than what is usually found for the purple bacterial systems ($\Delta G = 100-150$ mV; Dutton & Prince, 1978). However, low driving forces for the donation reaction seem to be a common property of RCI-type photosystems, since comparably low values have been determined for green sulfur bacteria (ΔG of about 50 mV; Prince & Olson, 1976) and PSI from chloroplasts ($\Delta G = 50$ mV; Drepper, 1994).

The donation reaction was found to proceed biphasically in a 1:1 stoichiometry of a faster (110 μs) and a slower (600 μs) phase under almost all experimental conditions, in line with previous work performed on whole cells by Vos et al. (1989). Only at ambient potentials above +200 mV, i.e. where both P_{798} and the electron-donating heme c_{553} are partially oxidized, were deviations from these kinetics found. A more detailed study of this effect is in progress.

As shown by our data, the *in vivo* kinetics are highly sensitive to disruption of cells. Depending on the degree of sample denaturation, kinetics for cytochrome oxidation with half-times between 6 and 20 ms can be observed. In fact, the heme which donates electrons under these conditions may even represent a different cytochrome. This, together with the observed, apparently nonphysiological reduction of heme c_{553} under certain redox conditions [see above as well as Kleinherenbrink and Amesz (1993)], indicates that membrane fragments are poor material for the study of secondary electron donation to P_{798}^+.

The kinetics of the donation reaction at saturating flash excitation and at very low flash intensities (i.e. with only about 10% of the total P_{798} photooxidized) were found to be similar within experimental error, showing that this kinetic phase is not limited by diffusion. It therefore seems to be determined by the intrinsic electron transfer time constant.

However, the reduction of the reaction center primary donor in heliobacteria is significantly slower than in other systems (where it ranges from hundreds of nanoseconds in purple bacteria to about 15 μs in PSI). In addition, we must consider the possibility that the rates for electron transfer among the cytochromes in the donor pool could be considerably more rapid. Therefore, the heme seen to be oxidized while P_{798}^+ is being rereduced may be different from the actual electron-donating redox species. Since all cytochromes found in heliobacteria so far appear to be membrane-bound (Trost & Blankenship, 1990), we favor the model of a complex between a protein containing one or more hemes c_{553} (see below) and the RC; the interaction between these two entities might be altered or even fully abolished upon disintegration of cells.

The biphasicity of the donation reaction remains enigmatic. The most obvious explanation invoking two different populations of reaction centers seems unlikely since, in all other respects, the population of RC's appears to be homogeneous (Trost & Blankenship, 1989; van de Meent et al., 1990; Liebl et al., 1993). Data reminiscent of the situation in heliobacteria have recently been reported for the purple sulfur bacterium *Ectothiorhodospira mobilis* (Leguijt, 1993). However, in this case, the biphasic donation was observed in the case where only the high potential pair of hemes contained in the RC-associated tetraheme subunit was reduced. Complete reduction of the cytochrome subunit restored monophasic kinetics as found in other purple bacteria (Leguijt, 1993).

The Cytochrome c_{553} Pool. As shown above, whole cells contain about 5−6 hemes c_{553} per RC. This pool of 553 hemes is heterogeneous as evidenced by the following sets of data. (a) A fraction of the full heme c_{553} content cannot be reduced by ascorbate (Figure 1). The electron-donating heme, however, titrates at +190 mV, i.e. is readily reduced by ascorbate. The fraction that cannot be reduced by

ascorbate is therefore distinguishable from the electron-donating heme(s). (b) Up to five different *c*-type heme proteins have been reported in heliobacteria, all of which show α-bands at or near 553 nm (Tros & Blankenship, 1990; Nitschke & Liebl, 1992; Albert & Michel, 1994). (c) At least four different hemes can be distinguished by EPR on membranes (N. Nitschke, B. Schoepp, B. Floss, W. A. Rutherford, and U. Liebl, unpublished results). In optical spectroscopy, all *c*-type hemes in these membranes were found to be characterized by α-bands at 553 nm.

In conflict with our data, Kleinherenbrink and Amesz (1993) and Vos et al. (1989) have reported that only less than 2 hemes can be photo oxidized per RC in membrane fragments and in whole cells of *H. mobilis*. However, as detailed in the Results, the redox state of the samples was not controlled in these experiments and they could therefore have been performed at rather high ambient potentials, leaving only a fraction of the full heme c_{553} complement reduced prior to the flash.

Two details of the heme oxidations induced by multiple flashes are noteworthy. (1) At least in the first three flash-induced donation reactions, P_{798}^+ was rereduced with similar kinetics. (2) The apparent E_m of the heme oxidized in the second flash was lower by about 20 mV as compared to that of the heme oxidized by the first flash, whereas the third flash heme oxidation titrated about 100 mV lower than the oxidations observed after the first two flashes.

The slightly differing E_m values for the first two flash oxidations can be rationalized in two different ways.

(a) Two or more electrochemically indistinguishable, noninteracting hemes c_{553} (either on different cytochromes or on one protein) are positioned at roughly similar distances with respect to P_{798}. Each of these hemes is able to donate an electron to the photooxidized special pair. This situation would result in differing apparent E_m values for the first and second flash oxidations (Case & Parson, 1971).

Such a model was proposed about 20 years ago for the case of purple bacteria containing RC-bound cytochromes. The structural features of the respective bound cytochrome in *R. viridis*, however, yielded an alternative interpretation for the respective experimental data [reviewed in Nitschke and Dracheva (1995)]. Notwithstanding, the hypothesis of several identical cytochromes might be applicable to the case of the heliobacteria. In fact, this model has been invoked recently to explain the donation reactions in green sulfur bacteria (Okumura et al., 1994).

(b) Alternatively, the difference in midpoint potential between the first and second flash oxidations may reflect the true individual E_m values of electrochemically differing hemes. Again, these hemes can be either on different cytochromes or part of a multiheme protein like in the case of most purple bacteria.

A decision between the different models has to await further progress with respect to biochemical characterization of the various cytochromes.

Irrespective of the choice of a model, however, the data demonstrate the presence of at least two hemes with very close or identical E_m values able to donate electrons either in parallel or in series to a common reaction center, in contrast to what has been proposed previously [e.g. see Kleinherenbrink and Almesz (1993)].

The heme oxidation following the third flash titrated at potentials far too low (+90 mV) to be rationalized in the framework of model a mentioned above. It therefore must represent a heme species distinguishable from the hemes titrating at about +170 to +190 mV.

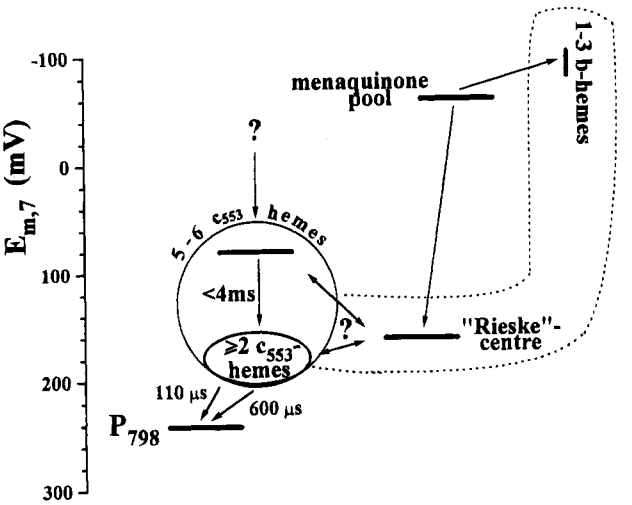

FIGURE 7: Schematic representation of the redox properties of components involved in rereduction of P_{798}^+ and the kinetic parameters for the respective electron transfer reactions as determined in this work. The amount of *b* hemes per P_{798} was estimated from Figure 1 and the total amount of photooxidizable c_{553} heme. A comparable value was obtained by direct observation of flash-induced absorption changes of cytochrome *b* hemes (Kramer, Schoepp, Floss, Liebl, and Nitschke, unpublished results).

Figure 7 schematically summarizes the kinetic and electrochemical data described above.

Rereduction of the Cytochrome c_{553} Pool. Rereduction of photooxidized heme c_{553} with physiologically relevant kinetics (i.e. in the range of hundreds of milliseconds) was found to occur only at redox potentials below +100 mV, corresponding to relatively anaerobic conditions within the cells. The major part of the reducing equivalents are providied from the menaquinone pool via the cytochrome *bc* complex (D. M. Kramer, B. Schoepp, B. Floss, U. Liebl, and N. Nitschke, unpublished results). The α-band of the *c*-type heme contained in the heliobacterial cytochrome *bc* complex was found at 554 nm (Nitschke & Liebl, 1992). Therefore, a fraction of the pool of c_{553} hemes actually must belong to the cytochrome *bc* complex. The virtually indistinguishable α-band wavelengths and the slow electron donation reaction to P_{798}^+ preclude a kinetic discrimination of the cytochrome *bc* contribution from the rest of the pool of cytochrome c_{553} hemes.

At potentials where the menaquinone pool is oxidized, the positive charge produced by the reaction center can therefore be transferred to the cytochrome *bc* complex without resulting in changes of the photoinduced heme spectrum. Only when the menaquinone pool becomes reduced (with an E_m value of about −60 mV; Liebl et al., 1992), delivery of electrons through the Q_0 site of the cytochrome *bc* complex into the pool of c_{553} hemes results in loss of the spectrum of oxidized heme c_{553}. The observed redox dependence of the onset of the reduction phases was consistent with this model. Furthermore, the velocities of the rereduction phases were in line with this process being limited by turnover of the Q_0 site (Velthuys, 1979; Crofts et al., 1983). The model outlined above is shown in Figure 7. Further studies using inhibitors affecting the cytochrome *bc* complex are expected to yield a more detailed picture of the

electron transfer steps between the cytochrome *bc* complex and the cytochrome(s) associated with the heliobacterial RC.

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