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Kinetic measurements of electron transfer in coupled chromatophores from photosynthetic bacteria

A method of correction for the electrochromic effects

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A quantitative study of the kinetics of electron transfer under coupled conditions in photosynthetic bacteria has so far been prevented by overlap of the electrochromic signals of carotenoids and bacteriochlorophyll with the absorbance changes of cytochromes and reaction centers. In this paper a method is presented by which the electrochromic contribution at any wavelength can be calculated from the electrochromic signal recorded at 505 nm, using a set of empirically determined polynomial functions. The electrochromic contribution to kinetic changes at any wavelength can then be subtracted to leave the true kinetics of the redox changes. The corrected redox changes of the reaction center measured at 542 and 605 nm mutually agree, thus providing an excellent test of self-consistency of the method. The corrected traces for reaction center and of cytochrome *b*-566 demonstrate large effects of the membrane potential on the rate and poise of electron transfer. It will now be possible to study the interrelation between proton gradient and individual electron transfer reactions under flash or steady-state illumination.

Electron transfer; Membrane potential; Bacterial photosynthesis; Electrochromism; Carotenoid; Bacteriochlorophyll

1. INTRODUCTION

Many attempts have been made to correlate the poise of electron transfer chains with that of the reactions of the ATP-synthase, proton gradient, or other 'energized state' associated with the coupling process. A complete description of the energy coupling process requires that such measurements be made in order that the results can be compared with hypothesis. In mitochondria the 'cross-over' points on transition from state 3 to state 4 have been interpreted as indicating sites of energy conservation, and the changes in redox poise of the electron transfer components associated with this transition have been estimated [1]. In addition, much work has been reported on apparent changes in E_m of redox couples on 'energization', for example on addition of ATP [1]. With the development of plausible models for the mechanism of coupled electron transfer, it seems worthwhile to reinvestigate the problem in the simpler systems represented by the photosynthetic bacteria. The cyclic electron transfer chains in chromatophores from *Rhodobacter capsulatus* and *Rb. sphaeroides* are essentially similar. Each is composed of a

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Abbreviations: BChl, bacteriochlorophyll; cyt, cytochrome; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); Mops, 4-morpholineethanesulfonic acid; PMS, *N*-methylphenazonium methosulfate; RC, reaction center

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photosynthetic reaction center which serves as both electron donor and acceptor to a ubiquinol:cyt c_2 oxidoreductase complex. Two diffusible electron carriers, ubiquinone and cyt c_2, allow redox coupling between these two complexes (reviews [2,3]). The kinetic and thermodynamic characteristics of these systems have been extensively studied in uncoupled chromatophores by kinetic spectrophotometry, exploiting the different redox spectra of many individual electron carriers [4–6], and have been successfully accounted for by a modified Q-cycle mechanism.

The photosynthetic pigments present in chromatophores undergo electrochromic color changes in response to electric fields in the membrane and provide a rapidly responding, linear 'membrane voltmeter'. This phenomenon has been extensively studied at the molecular level and has been used to monitor the transfer of charge across the membrane during electron transfer, and the bioenergetics of photophosphorylation (reviews [7,8]). The electrochromic responses of pigments are particularly prominent for carotenoids but also occur for the BChl bands at 590 [9] and 850 nm [10,11].

The ability to measure the membrane potential on a rapid time scale has been of great importance in identifying reactions of the electron transfer chain linked to electrogenic processes. However, the presence of electrochromic phenomena has hampered study of the kinetics of electron transfer in coupled chromatophores, because of the extensive overlapping of the electrochromic signals with the absorbance changes due to redox events [9]. Previous studies have used carotenoidless mutants where the interference from BChl electrochromism was not eliminated [12], or else carotenoid-containing strains where the interference from both carotenoid and BChl was ignored [13–15]. Here, we describe a general method by which the electrochromic contribution can be evaluated at any wavelength, and then used to correct the traces obtained in coupled chromatophores at the same wavelength. This method makes it possible to study the kinetics and thermodynamic poise of all the spectrophotometrically detectable components of the electron transfer chain and to compare these with the voltages and currents across the membrane associated with the generation and decay of the proton gradient.

2. MATERIALS AND METHODS

A green strain of *R. capsulatus* was grown photoheterotrophically in a synthetic malate-containing medium at high light intensity ($60\ W \cdot m^{-2}$). Chromatophores were prepared by French press disruption and differential centrifugation, as described [16]. BChl was evaluated according to Clayton [17].

Kinetic spectrophotometric measurements were performed in an N_2 atmosphere under controlled redox conditions as in [18]. All measurements were performed in 100 mM KCl, 50 mM Mops, pH 7, containing 2 μM nigericin and the redox mediators specified in [19]. The kinetic signals were averaged utilizing an M24 Olivetti personal computer interfaced to a DataLab DL905 transient recorder and stored on disk. Data analysis was performed by means of BASIC routines running on the same computer.

3. RESULTS

3.1. *Correction for the electrochromic changes*

The complex absorbance changes detectable upon light activation of coupled chromatophores in the spectral region 500–610 nm are associated with overlapping reduced-oxidized spectra of the redox carriers, and spectra of electrochromic effects of the photosynthetic pigments. In principle, all the electrochromic signals should be related to the value of the membrane potential ($\Delta\psi$), which can be monitored at a wavelength at which the electrochromic signal is large and not interfered with by redox changes. It should therefore be possible to compute the time course of the electrochromic signals at any other wavelength from this purely electrochromic trace, provided that appropriate normalizing factors are obtained from a complete spectrum of the electrochromic phenomena in the spectral region of interest. The calculated electrochromic contribution could then be subtracted from the measured trace to leave only the contribution from redox changes.

This simple logic is complicated by the nature of the electrochromic phenomena. These are characterized by a progressive shift to the red of the spectral bands of the pigments with increasing $\Delta\psi$. When the absorbance change associated with

the band shift is measured at the peak of the difference spectrum, the amplitude is proportional to the shift, which is proportional to the applied field. However, because the difference spectrum results from a shift, the changes are nonlinear with membrane potential at certain wavelengths. This nonlinear behavior should be most obvious in the wavelength range at which the light-dark difference spectrum of the electrochromic effect crosses zero [10]. The difference spectrum for the electrochromic change of the carotenoids in this strain of *Rb. capsulatus* was maximal at 505 nm. At this wavelength, contributions from absorption changes of redox components were 50-fold smaller than the maximal amplitude of the electrochromic response. The absorbance changes should therefore be linear with membrane potential, as previously observed [10]. The linearity of ΔA_{505} with $\Delta\psi$ is confirmed in the present work by the observation that, under conditions in which the only electrogenic reaction is the charge separation linked to oxidation and reduction of the reaction center, and the flash-oxidized components are rapidly and completely re-reduced by artificial electron donors, an identical rapid phase of the absorption change is produced on each flash in a train of 8 flashes, spaced 20 ms apart (fig.1, trace a).

For any wavelength λ, the correlation between ΔA_λ (electrochromic) and ΔA_{505} must be determined. A complete set of traces in the wavelength span 505–610 nm has been obtained, under conditions in which the electrochromic changes were maximized, and redox-related absorbance changes were minimized. In order to achieve this, turnover of the ubiquinol:cyt c_2 oxidoreductase (bc_1 complex) was prevented by inhibition with antimycin and myxothiazol, and DAD and PMS were added as artificial donors to cyt c_2. The chromatophore suspension was poised at an ambient redox potential of 120 mV. Under these conditions, the ubiquinone pool was largely oxidized and the added DAD reduced, and illumination by a train of 8 flashes activated a non-cyclic electron transfer from $DADH_2$ to ubiquinone, through the following sequence of reactions.

$$2P_{870}\cdot Q_A \longrightarrow 2P^+_{870}\cdot Q^-_A \quad (1)$$

$$2\text{cyt } c_2 + 2P^+_{870}\cdot Q^-_A \rightleftharpoons 2\text{cyt } c_2^+ + 2P_{870}\cdot Q^-_A \quad (2)$$

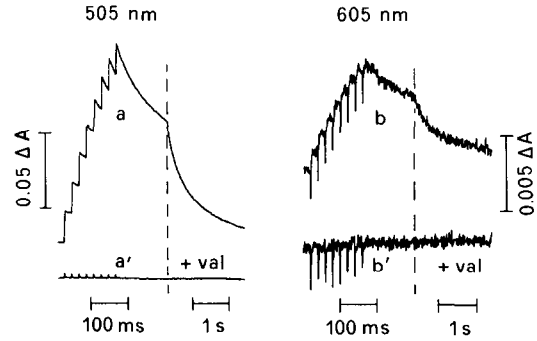

Fig.1. Absorption changes induced by a train of 8 single-turnover flashes, fired 20 ms apart, at 505 and 605 nm, in the absence (a,b) and presence (a',b') of 20 μM valinomycin. Assay conditions are described in section 2. The secondary electron transport was completely inhibited by 10 μM antimycin A and 3 μM myxothiazol. 0.5 mM DAD and 20 μM PMS were added as artificial electron donors. The ambient redox potential (E_h) was 120 ± 5 mV. Each signal was recorded at two different sampling rates: the dashed lines indicate the sweep change-over point. The instrument response time was 500 μs. The traces at 505 and 605 nm are an average of two and 8 measurements, respectively.

$$DADH_2 + 2\text{cyt } c_2^+ \rightleftharpoons DAD_{ox} + 2\text{cyt } c_2 + 2H^+ \quad (3)$$

$$2P_{870}\cdot Q^-_A + Q_{pool} + 2H^+ \rightleftharpoons 2P_{870}\cdot Q_A + QH_{2pool} \quad (4)$$

Reactions 1 and 2 in this sequence contribute to charging the membrane, with a total of one charge transferred per reaction center. The conditions were chosen so as to minimize the following potential artifacts:

(i) At E_h = 120 mV, both *b*-type cytochromes of the bc_1 complex were oxidized. This ensured that no electrophoretic redistribution of charge within the *b*-cytochrome chain could occur [20]. When cyt *b*-561 was reduced before illumination, an uncoupler-sensitive reduction of cyt *b*-566 occurred (Chen, Y. and Crofts, A.R., unpublished).

(ii) An excess of DAD was added to ensure rapid rereduction of cyt c_2 after each flash. This allowed for stoichiometric turnover of the system on each of the 8 flashes. It also ensured that reduction of both cyt c_2 and P^+_{870} was complete, despite the change in effective equilibrium constant for electron transfer from cyt c_2 to P^+_{870} resulting from the work against the membrane potential:

$$K_{eff} = K_{eq} \cdot \exp(-\alpha F \Delta\psi / RT)$$

(approx. $70 \times 10^{(-\alpha\Delta\psi/60)}$ where $\Delta\psi$ is in mV)

where α is the proportion of the electrical work due to reaction 2. Such a change in equilibrium constant has been extensively studied, both in intact chromatophores, and in vitro [21,22]. In the present work, the same phenomenon was reflected in the fact that the amplitude of cyt c_2 oxidation observed became smaller after each flash as the membrane potential increased (not shown).

Under these conditions, the only significant redox-linked absorbance changes were those due to P_{870}/P_{870}^+ and ferro-ferri cyt c_2 transitions, and these relaxed completely in the 20 ms between flashes. The electrochromic nature of the residual absorbance changes was demonstrated by addition of excess valinomycin, and traces in which the electrochromic effect contributed the only significant component were obtained by subtraction of the signal recorded in the presence of valinomycin (fig.1). In order to ensure minimal contributions from membrane potential-linked redox changes such as those discussed above, the traces were recorded on a dual time base, and included the full kinetics of decay over a time range of 2 s, i.e. 2 orders of magnitude longer than the relaxation of the observed redox reactions. The traces so obtained included a majority of points from the decay portion of the curve. When the points were plotted vs the corresponding time points from the trace obtained at 505 nm, with appropriate adjustment of scales, the nonlinearity of the electrochromic response at wavelengths away from the maximum of the difference spectrum could be seen from the departure from a straight line (fig.2). However, points from both the rising and decaying portions of the trace fell on the same line. In all cases, the points could be well fitted by a homogeneous polynomial function; although the computer routine could use polynomials up to the 5th degree, a suitable fit was found with a homogeneous quadratic polynomial, of the form

$$\Delta A_\lambda = P_1 \cdot \Delta A_{505} + P_2 \cdot \Delta A_{505}^2$$

Using the procedure illustrated above and in figs 1 and 2, we have obtained polynomial coefficients for a set of wavelengths suitable for the measurement of redox changes in the α-band region of the

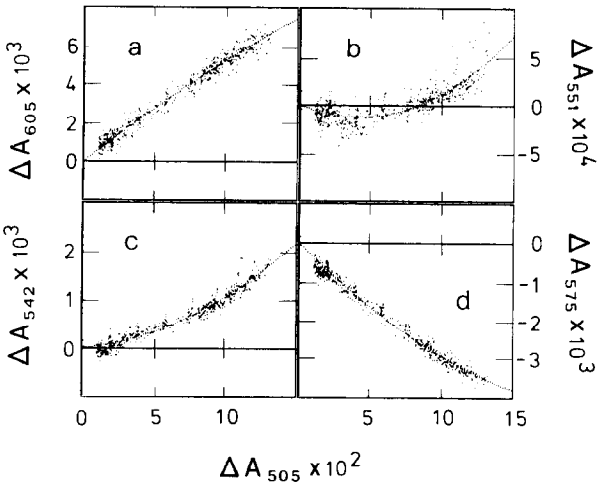

Fig.2. The correlation between ΔA_λ(electrochromic) and ΔA_{505} at λ = 605 nm (a), 551 nm (b), 542 nm (c) and 575 nm (d), obtained from traces analogous to those shown in fig.1. Each of 512 values of the valinomycin-sensitive ΔA sampled at wavelength λ was plotted vs the corresponding values of the valinomycin-sensitive ΔA sampled at 505 nm. Continuous lines give the calculated quadratic-polynomial fits for $\Delta A_\lambda = p_1 \cdot \Delta A_{505} + p_2 \cdot \Delta A_{505}^2$. Values obtained for coefficients p_1 and p_2: $p_1 = 5.88 \times 10^{-2}$, $p_2 = -5.93 \times 10^{-2}$ at 605 nm; $p_1 = -5.98 \times 10^{-3}$, $p_2 = 7.20 \times 10^{-2}$ at 551 nm; $p_1 = 2.82 \times 10^{-2}$, $p_2 = 7.71 \times 10^{-2}$ at 542 nm; $p_1 = -3.71 \times 10^{-2}$, $p_2 = 7.82 \times 10^{-2}$ at 575 nm.

spectrum. With appropriate precautions, the set of polynomial coefficients obtained under these special conditions can be used under any other conditions to find the electrochromic contribution at any of these wavelengths, by substitution in the equations above. By subtraction of the calculated electrochromic contribution, the changes due to redox reactions can be found, and deconvoluted by the well-established methods previously worked out under uncoupled conditions. It is therefore possible to study the dependence of electron transfer on the proton gradient under a wide variety of conditions and to measure simultaneously the thermodynamic poise and kinetics of the individual redox reactions, and of the proton gradient with which they are coupled.

Spectra of the electrochromic absorbance changes, constructed from the polynomial fits, are shown in fig.3. Traces at 505 nm of different amplitude, corresponding to three different values

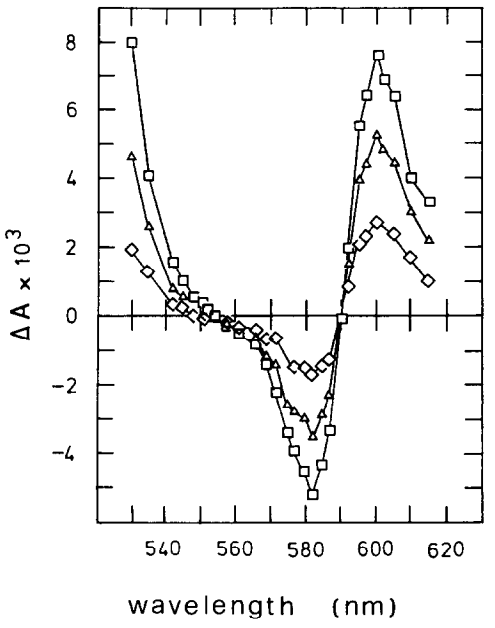

Fig.3. Spectra of the light-induced absorbance changes sensitive to valinomycin. The values of ΔA_λ were calculated from the quadratic-polynomial fitting the correlation between ΔA_λ and ΔA_{505} for three selected values of ΔA_{505}: (◇—◇) $0.043\Delta A$; (△—△) $0.086\Delta A$; (□—□) $0.129\Delta A$. These three values of ΔA_{505} correspond approximately to $\Delta\psi$ = 125, 250 and 375 mV, respectively, as judged by comparison with ΔA_{505} induced by a single-turnover flash in the presence of antimycin A and myxothiazol [27].

for $\Delta\psi$, were used as reference. The calculated spectra exhibit a marked red shift of the zero-crossing point around 550–552 nm at increasing $\Delta\psi$, as previously documented by other authors for the absorption bands of the carotenoids [23–26] and show the red-shifted band of BChl described by Bowyer and Crofts [9] in *R. capsulatus*. The absence of any detectable change in the position of the zero-crossing point at 590 nm is consistent with the small amplitude of the differential electrochromic BChl spectrum. Although small, these BChl electrochromic absorption changes overlap extensively, and are comparable in amplitude, with the light-induced redox changes in the α-band of *b*-type cytochromes, and in the 605 band of P^+_{870}. Likewise, the tail of the carotenoid band in the 540 nm region interferes with the signal of P^+_{870} and *c*-type cytochromes [9].

3.2. *Applications of the correction procedure*

Two examples of the correction procedure described above are presented. Traces at 542 and 605 nm for the light-induced redox changes of P_{870} were recorded in coupled chromatophores (fig.4a,a′); in both cases the signals are evidently distorted by electrochromic phenomena. The contributions from electrochromic effects (fig.4b,b′) were evaluated from the polynomial correlation coefficients measured at these two wavelengths and from a reference electrochromic trace recorded at 505 nm under identical experimental conditions. Although the calculated electrochromic contributions differ in amplitude and overlap redox changes of opposite sign, after subtraction the two corrected traces (fig.4c,c′) are kinetically similar and present a constant amplitude ratio of 1.75, in good agreement with the reported extinction coefficient ratio of the P^+_{870}/P_{870} difference spectrum at these wavelengths [4,28]. These corrected traces, when compared with the analogous ones recorded in the presence of valinomycin, demonstrate dramatic effects of the membrane

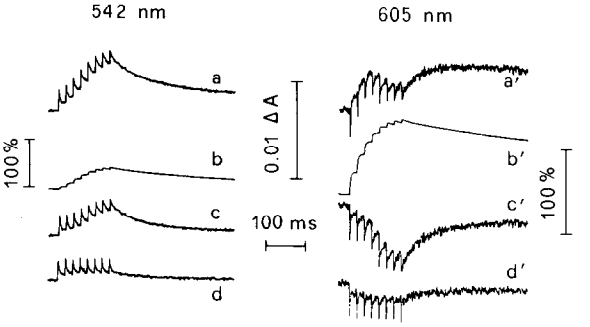

Fig.4. Kinetics of photooxidation and dark re-reduction of RC measured at 542 and 605 nm, corrected for electrochromic effects. Traces: (a,a′) crude experimental signals; (b,b′) electrochromic effects calculated from a 505 nm trace recorded under identical conditions (not shown) on the basis of the quadratic-polynomials specified in fig.2a,c; (c,c′) the two experimental traces corrected for electrochromic phenomena; (d,d′) traces from the same sample supplemented with 10 μM valinomycin. The 100% vertical bars indicate the maximum extent of ΔA_{542} and ΔA_{605} induced by the train of flashes in the same sample in the presence of 10 μM valinomycin and 10 μM antimycin. Traces at 542 and 605 nm are an average of 4 and 16 measurements, respectively. Sweep, 500 ms; RC, 200 μs. E_h = 120 ± 5 mV.

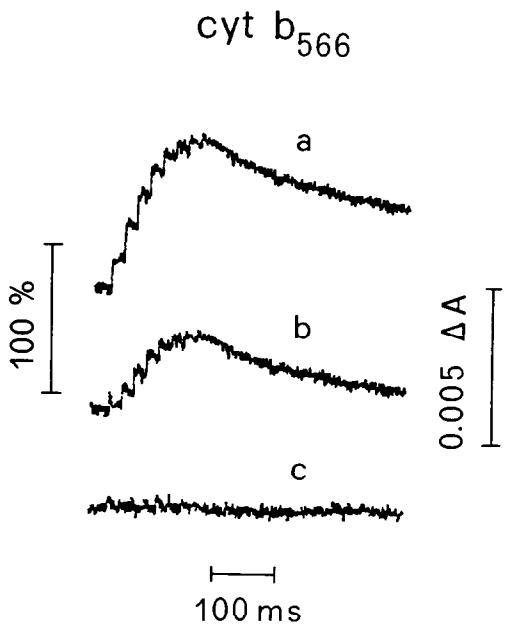

Fig.5. Kinetics of cyt *b*-566 in coupled and uncoupled chromatophores following a train of 8 flashes. Trace a: combination of four uncorrected traces recorded at the wavelengths suggested for cyt *b*-566 detection ($\Delta A_{566} - \Delta A_{575} - 0.5 \times (\Delta A_{561} - \Delta A_{569})$). The same combination was calculated for the four electrochromic effects evaluated from the appropriate quadratic-polynomials and subtracted from trace a: the result is shown in b; c: trace from the same sample supplemented with 10 μM valinomycin. The 100% vertical bar indicates the maximum extent of cyt *b*-566 reduction measured in the presence of 10 μM valinomycin and 10 μM antimycin. Signals are averages of 16 measurements. Sweep 500 ms, RC 200 μs. E_h = 120 ± 5 mV.

potential on the thermodynamic poise and the rate of re-reduction of P_{870}^+. A similar correction procedure was applied for the four wavelengths (566, 575, 561, 569 nm) suggested in [5] for the detection of cyt *b*-566 in the related species *R. sphaeroides* Ga. Again the trace obtained by deconvolution of the four uncorrected signals (fig.5a) differs very markedly from that obtained after subtraction of the calculated electrochromic contributions. The corrected trace (fig.5b) suggests an accumulation of reduced cyt *b*-566 in coupled chromatophores during a train of flashes, a phenomenon which is totally absent in uncoupled membranes (fig.5c) (cf. [5]). This accumulation is consistent with the proposed charge-separating steps within the bc_1 complex and the topology suggested for cytochromes *b* in the membrane [29].

4. DISCUSSION

The correction method described here assumes that the complex kinetic traces obtained in coupled chromatophores upon light activation of electron flow can be resolved as the sum of contributions from different redox changes and electrochromic signals. This logic has previously been used in uncoupled membranes to resolve the contribution of a specific redox carrier when reduced-oxidized differential spectra for more than one component overlap, and is here extended to coupled membranes. To this end, the reasonable assumptions needed are that the difference spectra of the redox carriers are not significantly distorted by the transmembrane electric fields, and that the electrochromic signal can be resolved as an independent component whose spectrum can be determined separately as a single-valued function of the membrane potential. Both of these assumptions seem to us quite acceptable.

We have chosen to fit the generally nonlinear dependence of ΔA_λ vs ΔA_{505} with a homogeneous polynomial and found that a quadratic curve is sufficient to describe the correlation with an excellent degree of accuracy. It should be stressed, however, that the function chosen and the parameters which define it at any wavelength do not necessarily have any fundamental physical meaning, but merely offer a phenomenological description of the correlation of ΔA_λ vs ΔA_{505}. For this reason, since the spectrum of the electrochromic signals depends on the pigment composition of chromatophores and on their electrochromic response, the set of constants for the fitting functions must be determined for every chromatophore preparation and set of experimental conditions. We are also aware of the danger of extrapolating the correlations on the basis of a pure phenomenological description; for this reason care has been taken to determine the fitting curves for an extent of membrane potential (i.e. of ΔA_{505}) similar to that generated in coupled membranes.

The reliability of the correction method described in this paper seems to be demonstrated in a very stringent test by the excellent agreement of the P_{870}^+ traces obtained at two different

wavelengths. A similar correction procedure applied at other wavelengths has yielded reasonable kinetics in response to the onset of $\Delta\psi$ for the other electron transport carriers (unpublished and cf. fig.5). However, it is of interest that the amplitude of the change in poise of cyt *b*-566 was rather less than that expected from the modified Q-cycle model, and the membrane potential indicated by the carotenoid change. The maximal value of the proton gradient to be expected from coupling to the bc_1 complex [30] is given by:

$$\Delta p_{max} = (E'_{cytc_2} - E'_{cytb\text{-}566})/2$$

In the experiment shown in fig.5, cyt *b*-566 was 55% reduced ($E' = -95$ mV, assuming $E_{m,7} = -90$ mV; $E' = -125$ mV if $E_{m,7} = -120$ mV) after 8 flashes when ΔA_{505} was maximal, and corresponded to a value for $\Delta\psi$ of about 350 mV assuming the calibration previously suggested [27]. Under the same experimental conditions, the extent of oxidation of P_{870} was about 75% ($E' = 478$, fig.4), representing the maximal oxidizing potential for cyt c_2. In fact, the potential of cyt c_2 was less than this because of the change in K_{eff} discussed above (not shown). Taking the E' values from above, and substituting, gives a maximal value for Δp of 287 mV (or 301 mV), well below the value indicated by the carotenoid change. Using a more realistic value for E'(cyt c_2) of about 400 mV, Δp_{max} becomes 248 mV (or 263 mV). Three possible causes for this discrepancy are under further investigation: firstly, the wavelength values used for the detection of cyt *b*-566 were derived from experiments using *Rb. sphaeroides*; this does not seem the most plausible explanation, since no major difference in the spectral properties of cytochromes was found in *Rb. capsulatus* [31]; secondly, some electrogenic reduction of cyt *b*-566 may have occurred despite the precautions taken, leading to an overcompensation of the electrochromic contribution; and finally, a component absorbing in the *b*-cytochrome region, but not part of the bc_1 complex, may have become oxidized, leading to an underestimation of the degree of reduction of cyt *b*-566. The lower values are more in line with values measured in other energy coupling systems, and by alternative methods for estimation of membrane potential, and suggest that the calibration of the carotenoid change may need revision. We are presently utilizing this approach for a systematic study of electron flow in the coupled state under flash or continuous illumination in *Rb. capsulatus* and *Rb. sphaeroides*.

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