Kinetic and thermodynamic resolution of cytochrome c_1 and cytochrome c_2 from *Rhodopseudomonas sphaeroides*

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1. INTRODUCTION

In chromatophores from *Rhodopseudomonas sphaeroides,* cytochrome *c* has several characteristics which suggest that there are two distinct populations. Among these are:

(i) A displacement of the α-band absorbance maxima of cyt. *c* 1–1.5 nm to the red in chromatophores relative to purified cyt. c_2 [1,2];

(ii) Intact spheroplasts and vesicles derived from them retain ~50% of the cyt. *c* found in cells or chromatophores but this cytochrome is unable to react rapidly with the reaction centers [2,3];

(iii) In the presence of UHDBT the cyt. *c* oxidation kinetics measured at 551–542 nm show two approximately equal phases with half-times of ~5–10 μs and ~200 μs [2].

Direct biochemical methods have shown that in both cells and chromatophores there are two separate *c* cytochromes, a bound cytochrome c_1 (λ_{max} 552 nm) and a soluble cyt. c_2 (λ_{max} 550 nm) [3,4]. Here, we present evidence on the kinetic, spectral and thermodynamic resolution of the two *c*-type cytochromes, and show that both are involved in cyclic electron transport, and function in series. A preliminary report of some of this work has appeared in [5].

2. MATERIALS AND METHODS

Chromatophores of *Rps. sphaeroides* were prepared as in [6] and suspended in buffer (50 mM MOPS, 100 mM KCl, pH 7.0) to ~0.2 μM reaction center. Time-resolved flash spectra and cytochrome kinetics were obtained as in [2]; the monochromator was a Hilger and Watts D330, and slits were set at 0.5 mm to give spectral bandwidth of 1.3 nm. Cytochrome c_2 kinetic changes were measured from the difference between the kinetics at 550 nm and 554 nm, and cyt. c_1 was measured at 552–548 nm. The flash redox titrations were performed as in [1,7]. Dark redox titrations were performed as in [8,9] and redox resolved spectra were obtained from full spectra redox titrations as in [10]. The spectrophotometer used a Hilger and Watts D330, D331 double monochromator, with slits set at 1 mm to give a bandwidth of 1.3 nm. UHDBT was a generous gift of B.L. Trumpower and the antimycin was obtained from Sigma.

3. RESULTS AND DISCUSSION

In fig. 1A spectra are shown of the two *c*-type cytochromes resolved by full spectra redox potentiometry. These spectra were obtained from a set of difference spectra measured at over E_h 200–400 mV, with the spectrum at 400 mV as reference. The differences in absorbance change at 551 and 542 nm as a function of E_h were abstracted from the data, and the resulting titration curve was used to find the best fit of components by computer analysis (see fig. 4 for typical results). For two $n = 1$ components, values for E_m of 257 and

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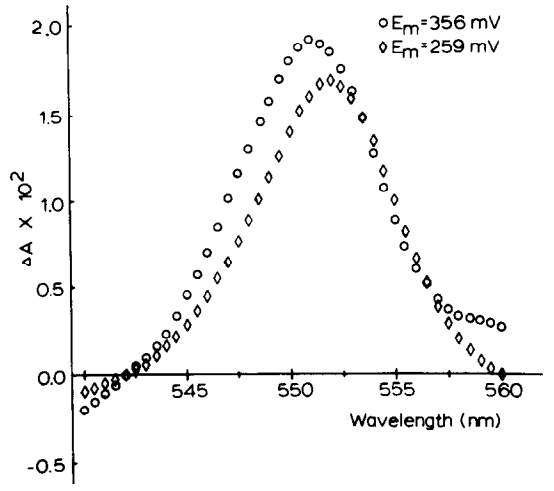

Fig. 1A. Chromatophores (~3 μM reaction center) were suspended in buffer (50 mM MOPS, 100 mM KCl, pH 7.0) and placed in a stirred anaerobic redox cuvette. The mediators present were: 100 μM benzoquinone, 100 μM DAD, and 500 μM ferri-ferrocyanide. Also present were 2 μM valinomycin and 1 μM nigericin. Spectra over the wavelength range from 515–579 nm were measured at 10 mV steps during a reductive and oxidative titration with similar results. The data from a reductive titration, using sodium dithionite as the reductant, was analyzed for 1, 2 or 3 components. The two component fit gave components with midpoints of 259 mV and 356 mV. These midpoints were used for further analysis to determine the absorption of each component at a series of wavelenghts and are plotted here.

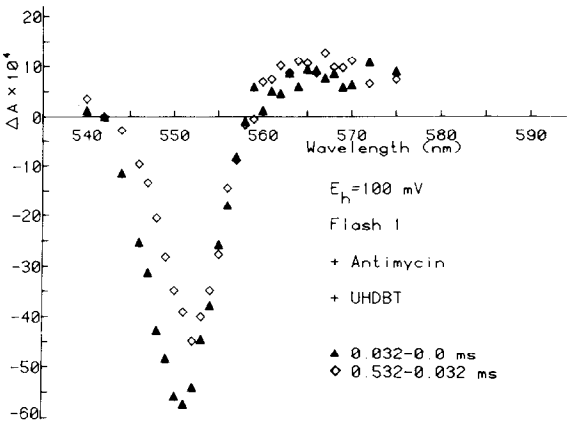

Fig. 1B. Chromatophores (~1 μM reaction center) were suspended in 100 mM KCl, 50 mM MOPS (pH 7.0) with the following mediators: 100 μM 1,2-naphthoquinone; 2 μM each of PMS, valinomycin and nigericin; 4 μg/ml gramicidin; 10 μM antimycin; and 40 μM UHDBT. They were poised at E_h 100 ± 10 mV in an anaerobic redox cuvette. Kinetic traces (av. of 16, 5 ms sweep, 10 μs filter RC, 30 s between flashes) were accumulated and stored at each point in the spectrum. The changes occurring over the times indicated give the spectra shown.

354 mV were calculated. Using these values, redox titration curves of the absorbance change as a function of E_h were obtained from the set of absorption spectra, for a series of wavelengths through the full spectral range (with the change at 542 nm as reference), and were fitted to find the contributions at each wavelength of the two components identified. The points in the figure show the contributions plotted against wavelength to give the separate spectra of the two components. Analysis of the set of data showed:

(i) The titration curve measured at 551–542 nm was fitted by two components better than by one component;

(ii) The λ_{max} of the two components were different (E_{m7} = 354 mV, α band, λ_{max} = 550.5 ± 0.5 nm; E_{m7} = 257 mV, α band, λ_{max} = 552 ± 0.5 nm);

(iii) In this preparation the components were present in approximately equal quantities (assuming similar extinction coefficients).

Similar titrations, but using various redox mediator concentrations, have consistently shown curves which are better fitted by two components than one, and have given E_{m7} values of 348 ± 13 mV, and 260 ± 11 mV (av. of 8 determinations).

In fig. 1B time-resolved spectra are shown of the flash-induced oxidation of the two *c* cytochromes, observed in the presence of UHDBT, to inhibit re-reduction by the Rieske-type FeS center [11,12] and corrected for the reaction center change [2]. The spectrum of the change occurring from just before the flash to 32 μs after the flash has a peak at 550 ± 0.5 nm. The change in absorption from 32–532 μs after the flash gives a spectrum with a peak at 552 ± 0.5 nm. Comparison of these spectra with that of purified cyt. c_2 and of cyt. c_1 in cyt. c_2-depleted membranes [4,13], shows that the component with λ_{max} at 552–553 nm is cyt. c_1.

From composite spectra obtained from several experiments of the sort shown in fig. 1, wavelengths were chosen to measure one *c*-type cytochrome while compensating for the change due to the other. The 'bound' cytochrome (c_1) can be measured at 552–548 nm. The 'free' cytochrome (c_2) can be measured at 550–554 nm. Fig. 2 shows the kinetics of the two cytochromes measured at these wavelengths, and the kinetics of the total cyt. *c* (measured at 551–542 nm), measured after flash excitation at several potentials in the presence of antimycin. It is clear from these traces that the two *c*-type cytochromes have distinctly different kinetics. Cytochrome c_2 is oxidized very rapidly ($t_{1/2} \sim 5\ \mu s$), while cyt. c_1 is oxidized much more slowly ($t_{1/2} \sim 150\ \mu s$). At potentials where the quinone pool is partly reduced ($E_h = 100$ mV) re-reduction of both *c*-type cytochromes can be seen on the ms time scale. At an $E_h = 200$ mV, where the quinone pool is oxidized, only cyt. c_2 is re-reduced rapidly, and at 300 mV neither of the *c*-type cytochromes becomes rapidly reduced. Fig. 3 shows the kinetics of the two *c*-type cytochromes in the presence of UHDBT, which inhibits re-reduction of the cytochromes by the Rieske-type FeS [11,12]. Although the kinetics show a greater oxidation and an inhibition of re-reduction, the half-times of oxidation remain essentially the same. The traces shown here demonstrate that UHDBT inhibits the re-reduction of both cyt. c_2 and c_1. Whether this inhibition is due simply to the effect of UHDBT in raising the E_m-value of the Rieske center or to a separate kinetic effect cannot be determined from these experiments.

The sequence of electron transfer reactions from the *c*-type cytochromes to the oxidation reaction center (P^+) can be firmly established from the kinetic results. Cytochrome c_2 is the reductant for

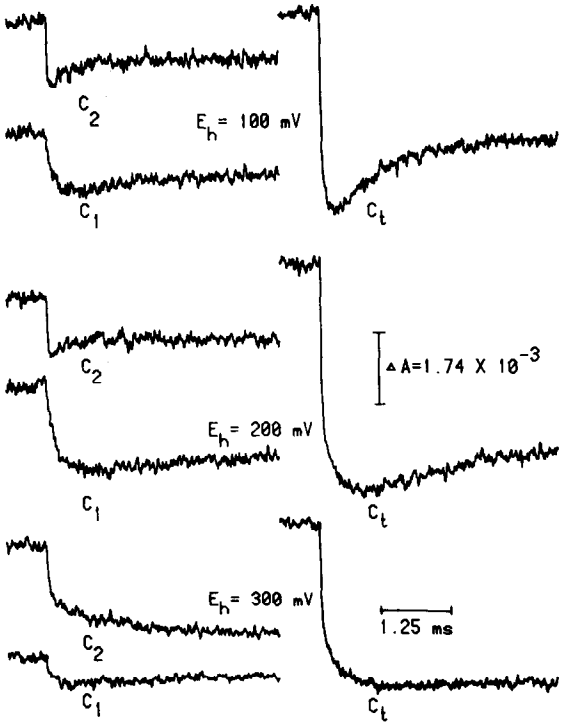

Fig. 2. Chromatophores were suspended to ~1 μM reaction center in 100 mM KCl, 50 mM MOPS (pH 7.0). Mediators were present in the following concentrations; at $E_h \sim 300$ mV, 100 μM benzoquinone, 1 μM TMPD; at $E_h \sim 200$ mV, 100 μM 1,2-naphthoquinone, 1 μM TMPD; at E_h ~00 mV, 100 μM 1,2-naphthoquinone, 1 μM TMPD, 1 μM PMS. Present at all potentials were antimycin (10 μM), gramicidin (4 μg/ml), valinomycin (2 μM), and nigericin (2 μM). Kinetic traces (av. of 8, 5 ms sweep, 10 μs filter RC) were accumulated at a set of wavelengths and stored for analysis. Cytochrome c_t ($c_1 + c_2$) was measured at 551 nm, cyt. c_1 was measured at 552–548 nm, and cyt. c_2 was measured at 550–554 nm. All changes were corrected for reaction center changes by subtracting the normalized 542 nm trace [2].

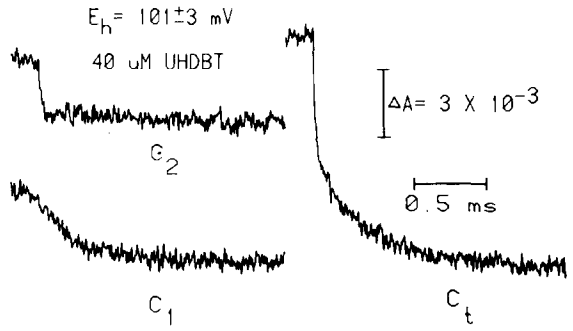

Fig. 3. Chromatophores were suspended to ~1 μM reaction center in 100 mM KCl, 50 mM MOPS (pH 7.0). The mediators present were 100 μM 1,2-naphthoquinone and 1 μM PMS. Also present were 1 μM nigericin, 2 μM valinomycin, 4 μg gramicidin/ml, 10 μM antimycin and 40 μM UHDBT. Kinetic traces (av. of 16, 2 ms sweep, 5 μs filter RC) were accumulated at a series of wavelengths and stored.

P^+ with a half-time for oxidation of ~5 μs. From the volume of the internal aqueous phase, and the number of molecules of reaction center and of cyt. c_2/chromatophore, a second-order rate constant of $\sim 2 \times 10^8$ $M^{-1}.s^{-1}$ can be shown to account adequately for this half-time, and this agrees well with a value measured using the purified components (8×10^8 $M^{-1}.s^{-1}$) [14]. Several groups [14–16] have suggested that an association between cyt. c_2 and the reaction center may occur, giving a first-order process for oxidoreduction in the complex, and the kinetics measured at high concentrations of the purified components show first-order characteristics. It is not clear whether the reaction is first or second order in situ; however, it is clear that cyt. c_2 is a necessary intermediate carrier between the ubiquinol:cyt. c_2 oxidoreductase complex and the reaction center, since the oxidation of cyt. c_1 following a flash [2] or continuous illumination [3] is greatly slowed when cyt. c_2 is extracted during preparation of spheroplasts or of vesicles derived from spheroplasts [2,3]. In view of the relative stoichiometries, 1 cyt. c_2 and 1 cyt. c_1 for 2 reaction centers [5], it seems clear that cyt. c_2 must leave its site of oxidation and cycle between the complex and the reaction center in order to complete the oxidation of both cytochromes, and the reduction of P^+.

While it is clear that cyt. c_1, and from the effects of UHDBT, also the FeS center, are oxidized via cyt. c_2, the relative positions of FeS and cyt. c_1 are more ambiguous. The most reasonable conclusion is that FeS is oxidized by cyt c_1, and that the high potential components are arranged in the series FeS–c_1–c_2–P. This arrangement is consistent with the kinetic behavior, and with the observation that UHDBT inhibits re-reduction of both *c*-type cytochromes. However, on the basis of the kinetic evidence we cannot exclude the possibility that FeS and cyt. c_1 operate in parallel. By analogy with the mitochondrial complex, such a parallel pathway seems unlikely, since in complex III the binding site for cyt. *c* on the complex is the same as that on isolated cyt. c_1 [17], and in the isolated complex, removal of FeS mimics the effects of UHDBT in preventing reduction of cyt. c_1 but not its oxidation [18]. If a parallel pathway does operate, it would be necessary to postulate that FeS and cyt. c_1 are in rapid equilibrium, since the kinetics of the UHDBT-sensitive reaction [19],

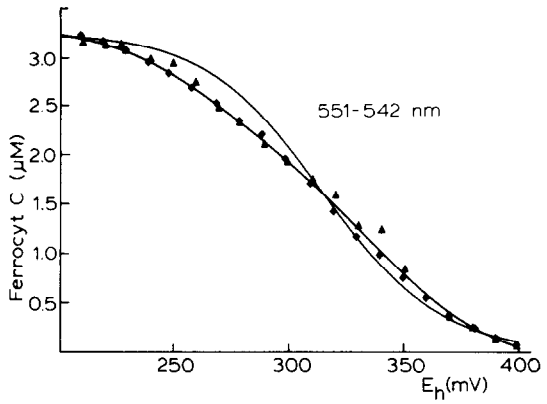

Fig. 4A. Chromatophores were suspended to ~3 μM reaction center in buffer (100 mM KCl, 50 mM MOPS, pH 7.0) in an anaerobic redox cuvette. The mediators present were: 100 μM benzoquinone, 2 μM valinomycin, 1 μM nigericin, 100 μM DAD, and 500 μM ferri-ferrocyanide. Spectra were scanned over the wavelength range from 515–579 nm at each E_h. The difference measured at 551–542 nm was normalized to the maximum change and plotted. Different symbols represent different experiments.

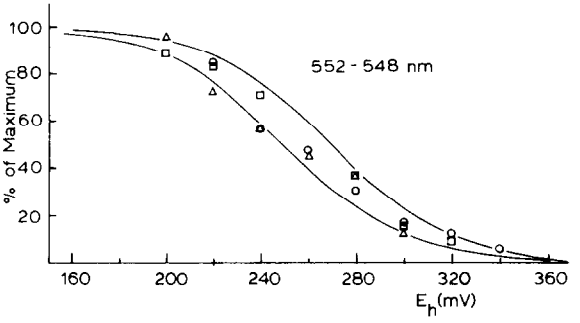

Fig. 4B. Chromatophores were suspended in 50 mM MOPS, 100 mM KCl (pH 7.0). Kinetic traces (av. of 4, 10 ms sweep, 20 μs filter RC) were averaged and stored. The extents of the cyt. c_1 oxidation were measured and analyzed for their midpoints. The extents were then normalized with the theoretical maximum being 100%, and plotted. The different symbols represent different experiments. Present in all experiments were 1 μM valinomycin and 2 μM nigericin: (△) mediators 1 μM TMPD, 100 μM ferri-ferrocyanide, 100 μM benzoquinone, theoretical E_{m7} = 256 mV; (○) 1 μM TMPD, 100 μM ferri-ferrocyanide, 100 μM benzoquinone and 40 μM UHDBT, theoretical E_{m7} = 252 mV; (□) 2 μM DAD, 2 μg gramicidin/ml, 100 μM ferri-ferrocyanide, theoretical E_{m7} = 268 mV.

cyt. c_1 oxidation and cyt. c_2 re-reduction all occur with half-times of 100–200 μs. Because of the stoichiometries of the components, the separate kinetic contributions of the different processes to the observed kinetics (fig. 2,3) cannot be simply resolved.

In fig. 4 a dark equilibrium redox titration is shown of the total *c* cytochrome measured at 551–542 nm and a redox titration of the flash induced oxidation of cyt. c_1 measured at 552–548 nm. Through the points from the dark redox titrations (fig. 4A) are drawn computer-generated one- and two-component curves with E_m 315 mV and 340 mV and 265 mV at relative concentrations of 62% and 38%, respectively. The points from the titration of cyt. c_1 oxidation (fig. 4B) show ~20 mV of hysteresis between oxidizing and reducing titrations. Drawn through the data are two theoretical one-component curves with midpoints of 250 and 270 mV.

4. SUMMARY

These results show that the 'bound' *c* cytochrome (c_1) is a functional component of single-turnover photosynthetic electron transport. The cytochrome appears to have a midpoint potential of 260 ± 11 mV as determined by flash and dark redox titrations. This cytochrome is apparently oxidized by cyt. c_2 and its re-reduction is inhibited by UHDBT. Whether the 'bound' cyt. *c* is reduced by the Rieske FeS center, or is reduced and oxidized in parallel with it cannot be determined from these results. The values for the midpoint potential of cyt. c_1 (E_m ~ 260 mV) from both sorts of experiments above are in contrast to higher values (E_m ~ 285–290 mV) reported in [4] using spheroplasts, and in [13] with a partially purified ubiquinol:cyt. c_2 oxidoreductase complex; we have also been able to measure a higher E_m-value (285 mV) for cyt. c_1 in spheroplasts (not shown). It seems possible that the differences between these values may reflect either the fact that in these experiments, the potential was measured in the presence of cyt. c_2, or that in the chromatophore system, the cyt. c_1 site is inside the vesicle.

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REFERENCES

[1] Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536–556.
[2] Bowyer, J.R., Meinhardt, S.W., Tierney, G.V. and Crofts, A.R. (1981) Biochim. Biophys. Acta 635, 167–186.
[3] Wood, P.M. (1980) Biochem. J. 192, 761–764.
[4] Wood, P.M. (1980) Biochem. J. 189, 385–391.
[5] Crofts, A.R., Meinhardt, S.W. and Bowyer, J.R. (1982) in: The Function of Quinones in Energy Conserving Systems (Trumpower, B.L. ed) pp. 477–498, Academic Press, New York.
[6] Bowyer, J.R., Tierney, G.V. and Crofts, A.R. (1979) FEBS Lett. 101, 201–206.
[7] Bowyer, J.R., Tierney, G.V. and Crofts, A.R. (1979) FEBS Lett. 101, 207–212.
[8] Evans, E.H. and Crofts, A.R. (1974) Biochim. Biophys. Acta 357, 78–88.
[9] Crowther, D. (1977) PhD Thesis, University of Bristol, Bristol.
[10] Meinhardt, S.W. and Crofts, A.R. (1982) submitted.
[11] Bowyer, J.R., Dutton, P.L., Prince, R.L. and Crofts, A.R. (1980) Biochim. Biophys. Acta 592, 445–460.
[12] Bowyer, J.R. and Crofts, A.R. (1981) in: Frontiers in Bioenergetics, vol. 1/pt 2, pp. 321–333, Academic Press, New York.
[13] Gabellini, N., Bowyer, J.R., Hurt, E., Melandri, B.H. and Hauska, G. (1982) Eur. J. Biochem. 126, 105–111.
[14] Prince, R.C., Cogdell, R.J. and Crofts, A.R. (1974) Biochim. Biophys. Acta 347, 1–13.
[15] Overfield, R.E. and Wraight, C.A. (1980) Biochemistry 19, 3322–3327.
[16] Overfield, R.E. and Wraight, C.A. (1980) Biochemistry 19, 3328–3334.
[17] Rieder, R. and Bosshard, H.R. (1980) 1st Eur. Bioenerget. Conf. short rep. pp. 87–88.
[18] Bowyer, J.R., Edwards, C.H. and Trumpower, B.L. (1981) FEBS Lett. 126, 93–97.
[19] Bowyer, J.R. and Crofts, A.R. (1981) Biochim. Biophys. Acta 636, 218–233.