EQUILIBRIUM AND DISEQUILIBRIUM IN THE UBIQUINONE-CYTOCHROME $b-c_2$ OXIDOREDUCTASE OF *RHODOPSEUDOMONAS SPHAEROIDES*

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1. Introduction

Electron flow through a quinone-cytochrome $b-c$ oxidoreductase is a common feature of energy-transducing electron transfer pathways, including those of mitochondria, chloroplasts and many species of bacteria. Photosynthetic bacteria, such as *Rhodopseudomonas sphaeroides*, provide a useful experimental system for the study of such electron flow, because single turnover flashes of light provide the ubiquinone-cytochrome $b-c_2$ ($UQ/b-c_2$) oxidoreductase with a single oxidizing and a single reducing equivalent within 1 ms of the flash. Using redox potentiometry in conjunction with rapid spectrophotometric analysis, we have measured the kinetics and stoichiometries of several important steps in the $UQ/b-c_2$ system, including the number of protons bound from the external aqueous phase per turnover, which has proven to be close to 2.0 H^+/e^- [1]. One of these protons, (H_I^+), is unaffected by antimycin, and is bound with a halftime of 100 μs commensurate with the emergence of the electron from the photochemical reaction center; the agent responsible for binding H_I^+ is thought to be a ubisemiquinone [2]. Only the second proton (H_{II}^+) is antimycin sensitive, but other details of the factors which govern the binding of H_{II}^+ are unclear.

We have also recently characterized the component, Z [3], which plays a central role in the $UQ/b-c_2$ oxidoreductase; it has an equilibrium redox reaction of $Z + 2e^- + 2H^+ = ZH_2$, with E_{m7} 155 mV [4,5]. In vivo, ZH_2 reduces flash-oxidized cytochrome c_2 in the ms time range in an antimycin-sensitive reaction (i.e., $ZH_2 + c_2^{ox} = c_2^{red} + Z^{\cdot}H + H^+$). The fate of $Z^{\cdot}H$ is unclear, but since it is unstable it is presumably either rapidly oxidized to Z, or reduced to ZH_2. Within the framework of current chemiosmotic models, the uptake of H_{II}^+ would be expected to depend on the fate of $Z^{\cdot}H$, and to have kinetics similar to those of the reduction of ferricytochrome c_2. With Z redox poised as ZH_2 before activation, so that flash-oxidized ferricytochrome c_2 is promptly reduced [4,5], H_{II}^+ binding is indeed in the ms time range [1]. However, when Z is poised oxidized before flash activation, H_{II}^+ binding is not only still evident, it is bound with a halftime of $\sim$ 200 μs [1]. This is much faster than any measured electron transfer event in the $UQ/b-c_2$ oxidoreductase, and if correct, indicates that current models for the system are inadequate.

Mitchell has made several suggestions regarding H_{II}^+ [6]. The $Q/b-c_2$ oxidoreductase of *Rps. sphaeroides* was proposed [6] to be similar to the $Q/b-c_1$ system of mitochondria in that the former should display oxidant-induced reduction of b-cytochromes [7,8]. In mitochondria provided with succinate and antimycin, the addition of ferricyanide induces an oxidation of cytochromes c and c_1 and a reduction of cytochromes b. In *Rps. sphaeroides* a similar reaction might involve the following obligatorily-coupled sequence:

$$\text{ferricyanide} + c_2^{red} \rightleftharpoons \text{ferrocyanide} + c_2^{ox}$$

$$c_2^{ox} + ZH_2 + b^{ox} \rightleftharpoons c_2^{red} + Z + b^{red} + 2H^+$$

Excess ferricyanide would reoxidize the c_2, leaving b

reduced and therefore out of direct equilibrium (i.e., $\Delta G \neq 0$) with the high redox potential exerted by the external ferri/ferrocyanide. If added redox dyes reacted exclusively with cytochrome c_2, then it is possible that in adjusting the redox potential so that Z is oxidized, *via* c_2, before activation, cytochrome *b* might become reduced and therefore possess an electron which could be immediately available for H^+ binding after flash activation. This report provides an experimental assessment of such a possibility.

2. Materials and methods

Chromatophores from *Rhodopseudomonas sphaeroides* strains R26 (blue–green mutant) and Ga (green mutant) were prepared as in [9]. Redox potentiometry [10] was used in conjunction with either a rapid dual wavelength spectrophotometer for the analysis of flash-activated reactions [9] or with a scanning dual wavelength spectrophotometer interfaced with a Digital PDP 11/10 computer for standard difference spectroscopy.

3. Results and discussion

Figure 1 is a series of difference spectra, obtained at pH 6, between different redox potentials. Redox mediators 2,3,5,6-tetramethylphenylenediamine (DAD, E_{m7} 240 mV) and *N*-methylphenazonium methosulfate (PMS, E_{m7} 80 mV) were present at 5 μM, typical for flash-activated experiments [1–5, 9–13]. Dithionite was added to reduce all the redox centers of the Q/*b*–c_2 oxidoreductase, and after a 20 min equilibration time a spectrum was taken. The E_h was below −130 mV. Ferricyanide was added to bring the E_h to 160 mV, then to 260 mV, and again to 470 mV. Reproducible spectra were obtained within 2–3 min of each adjustment of the redox potential, and the 470 mV was maintained for 30 min with no further cytochrome oxidation. The ΔA from −130–160 mV represents the oxidation of cytochromes b_{-90} (E_{m6} −30 mV) and b_{50} (E_{m6} 110 mV) (see [11]). The ΔA from 160–470 mV represents the oxidation of cytochrome b_{155} (E_{m6} 215 mV), and c_2 (E_{m6} 300 mV). The former was oxidized from

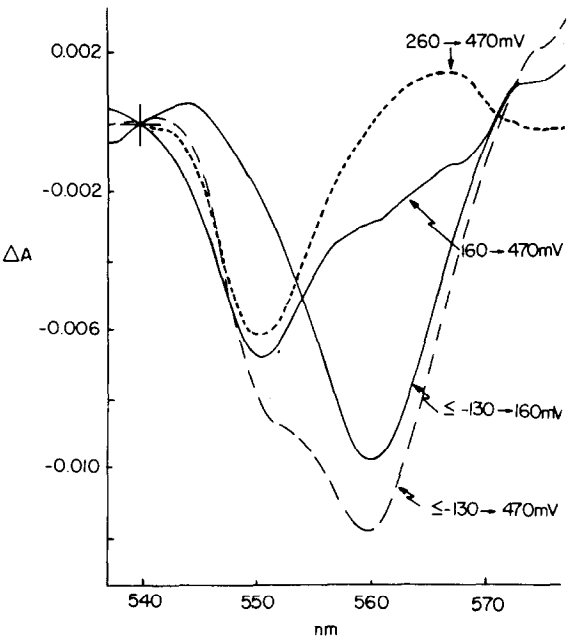

Fig.1. The *b* and *c* cytochromes of *Rps. sphaeroides*. Chromatophores of the Ga mutant (220 nM reaction centers) were suspended in 100 mM KCl, 20 mM 2(*N*-morpholino) ethane sulfonate, 1 mM $MgCl_2$, pH 6.0 in the presence of 5 μM PMS and DAD. The ambient potential was first adjusted to below −130 mV, and the chromatophores maintained at this potential for 20 min. The E_h was then raised sequentially to 160 mV, 260 mV and 470 mV. Spectra were recorded at each E_h and stored in the computer memory. The different spectra between pairs of these spectra are plotted in the figure.

160–260 mV, the latter from 260–470 mV. Figure 1 thus indicates that at pH 6, where the kinetics of H^+_{II} were determined [1], neither cytochrome b_{-90} or b_{50} will be found unexpectedly reduced at potentials above 160 mV, nor any *b*-cytochrome at potentials of 260 mV or above, if 2 or 3 min are allowed for equilibration.

Further confidence in this conclusion is found in fig.2A, which shows the time course of ferricyanide-induced reactions of cytochrome c_2 (553–540 nm) and *b* (559–540 nm). In these experiments only DAD was present as a mediator, and the E_h was poised at ~ 185 mV before a small addition of ferricyanide transiently raised the potential to 300 mV. The E_h then dropped back to 215 mV as

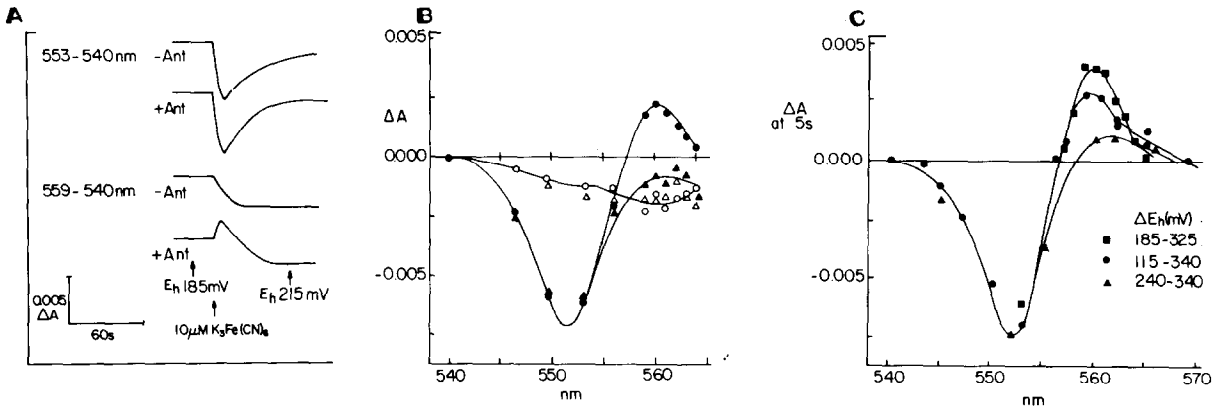

Fig.2. Oxidant-induced reduction of cytochrome *b* in *Rps. sphaeroides*. Chromatophores of the R26 mutant (20 μM BChl) were continuously stirred in 200 mM mannitol, 50 mM sucrose, 50 mM *N*-morpholino propane sulfonate, pH 7.0, in the presence or absence of 5 μM antimycin. (A) shows the kinetics of the cytochrome changes seen when the E_h is raised from 185–215 mV, and (B) shows spectra of these changes at 5 s (●▲) and 60 s (○△) in the presence (●○) or absence (▲△) of antimycin. DAD, 20 μM, was present in these experiments. (C) shows 5 s spectra measured in the presence of antimycin when the E_h was raised from 185–325 mV (■) in the presence of 20 μM PMS, from 115–340 mV (●) in the presence of 10 μM PMS, and from 240–340 mV (▲) in the presence of 8–24 μM DAD.

the DAD reduced the ferricyanide. Figure 2B shows that cytochrome c_2 underwent oxidation and reduction in concert with these changes, indicating that it was in equilibrium with the ambient E_h, regardless of the presence or absence of antimycin. In the absence of antimycin there was no detectable reduction of cytochrome *b*, and the net *b* oxidation was probably that of b_{155} which was partially reduced at 185 mV; the higher $A_{560-570}$ values at 5 s are those expected for the transiently oxidized c_2. However, in the presence of antimycin there was a transient but clear reduction of a *b*-cytochrome. It was reduced after 5 s but reoxidized by 60 s, at which time the spectrum was the same as that seen in the absence of antimycin. The 5 s spectrum of cytochrome c_2 oxidation and *b* reduction is very similar to the flash-induced spectra in [13].

Figure 2C shows spectra recorded 5 s after a larger addition of ferricyanide, sufficient to maintain final $E_h \sim 340$ mV, when the chromatophores had been poised at various initial potentials. With starting E_h 240 mV, where Z is fully oxidized, only cytochrome c_2 oxidation was observed. A spectrum similar to that of fig.2B was obtained starting at 185 mV, and despite the higher final E_h the decay of the transient *b* reduction was stimulated by a factor of only 2 or 3. The transient reduction of a *b*-cytochrome was still seen at starting E_h 115 mV, although in this case the smaller extent was probably due to the simultaneous oxidation of b_{155} (fig.1). No transient *b* reduction was detected if the initial potential was 0 mV so that cytochrome b_{50} was reduced before activation, although this could possibly indicate an equally prompt and coincident oxidation of cytochromes b_{50} and b_{155}.

The data of fig.2 can be reproduced at pH 6 or 7 with both the Ga and R26 mutants, and they can be obtained with a variety of mediators. For example at E_h 180 mV (pH 7.0) they can be reproduced using either PMS, which is essentially oxidized at this potential, or DAD, which is mainly reduced. Increasing the mediator concentration in the 4–25 μM range had little effect on the appearance of the oxidant-induced reduction, which could still be observed in the presence of the full mediator complement usually used in kinetic experiments (~ 10 μM each of DAD, PMS, *N*-ethylphenazonium ethosulfate (PES, E_{m7} 55 mV) pyocyanine (E_{m7} –83 mV) and 2-hydroxy-1,4-naphthaquinone (E_{m7} –145 mV) see [1–5, 9–13]. However higher concentrations of mediators stimulated the relaxation of the *b* reduction, until at 50 μM it could not be detected with the slow

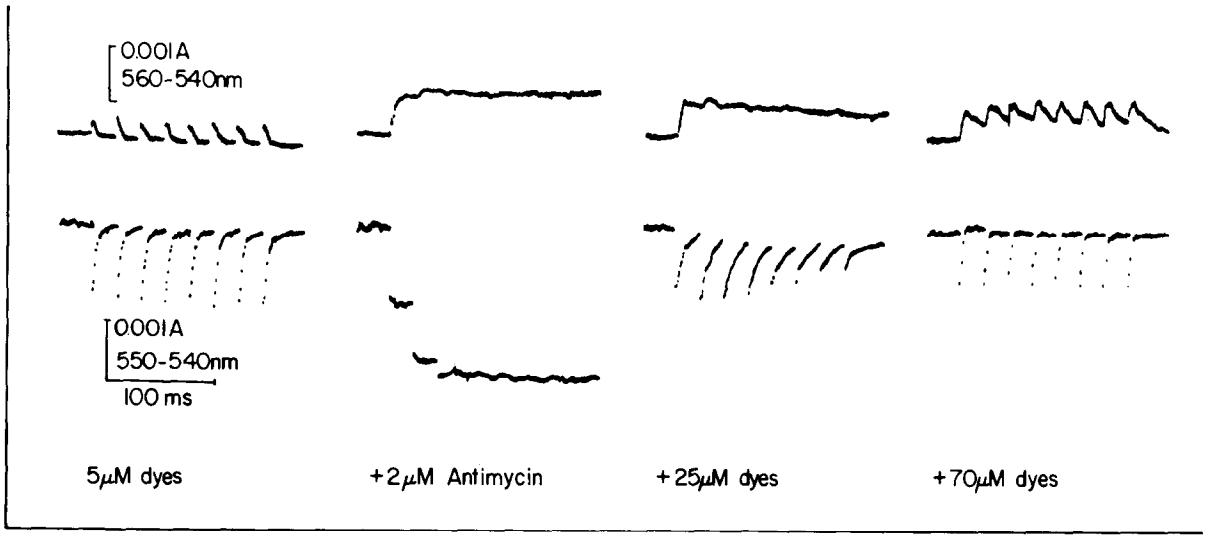

Fig.3. The effects of redox mediators on the kinetics of cytochrome changes in *Rps. sphaeroides*. Chromatophores of the Ga mutant (120 nM reaction centers) were suspended in 100 mM KCl, 20 mM *N*-morpholino propane sulfonate, 1 mM $MgCl_2$, pH 7.0, with 10 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 2 μM valinomycin to remove any energetic constraints from the coupling mechanism. PMS, PES, 2-hydroxy-1,4-naphthaquinone, DAD and pyocyanine were added as redox mediators as indicated; the additions shown are sequential. The traces are the average of 8 pulse sequences separated by 40 s.

resolution time used here. In agreement with this observation, such high concentrations of mediators interfered with electron flow on a rapid timescale (fig.3). On the left are typical kinetics of flash-induced oxidation–reduction of c_2 and reduction–oxidation of b_{50} observed when uncoupled chromatophores are poised at E_h 100 mV (c_2, Z and b_{155} reduced; b_{50} oxidized). Kinetics such as these are readily observed in appropriately anaerobic chromatophores in the absence of redox mediators [4]. Addition of antimycin dramatically slowed both c_2 re-reduction and *b* re-oxidation after each flash [13], and relaxation under these conditions takes seconds. An additional 25 μM of each of the mediator dyes obviously increased the relaxation of both cytochromes, although more dramatically with c_2; a total of 100 μM dyes caused the entire system to relax almost completely within 50 ms.

From the foregoing, chromatophores poised at high potentials would not be expected to reveal a flash oxidation of cytochrome *b* because all the cytochrome will be oxidized in equilibrium with the ambient E_h. Figure 4 shows spectra measured 500 μs after a single turnover flash, because this would be appropriate if the oxidation of a *b*-cytochrome were responsible for the 200 μs H^+_{II} binding. The spectra are very similar to those measured in the presence of antimycin [9]. In neither case can the small very rapid $A_{560-540}$ decreases be ascribed to a cytochrome *b*

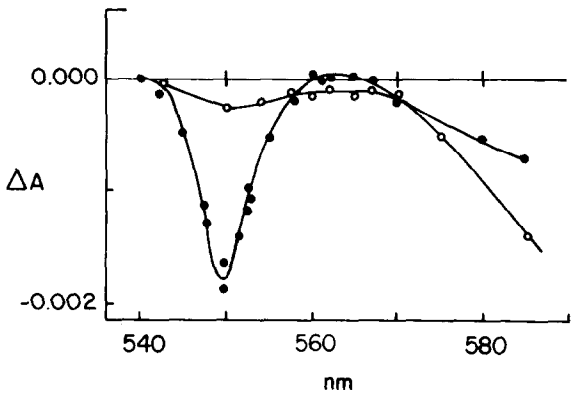

Fig.4. Flash-induced cytochrome changes in *Rps. sphaeroides*. Chromatophores of the Ga mutant (126 nM reaction centers) were suspended in the medium of fig.1, and the potential was poised at 260 ± 8 mV (•) and 388 ± 2 (○). Δ*A* was measured 500 μs after the flash.

oxidation: instead they are clearly due to reaction center changes, together with cytochrome c_2 oxidation at E_h 240 mV.

The following conclusions can be drawn:

1. Ferricyanide induced reduction of cytochrome *b* cannot be observed at room temperatures in the absence of antimycin, even with very low concentrations of redox mediators.

2. The ferricyanide induced reduction of cytochrome *b* is readily seen in the presence of antimycin, and is collapsed only by rather high levels of mediator dyes. It is seen only if a component with an E_{m7} significantly below 240 mV is reduced; a probable candidate for this component is Z [3–5]. It is still seen if b_{155} is reduced before the addition of ferricyanide, but not if b_{50} is also reduced. With the proviso discussed above, this suggests that b_{50} is the *b*-cytochrome undergoing reduction, and the spectral characteristics (fig.1,2) are consistent with this identification.

3. The experiments reported here support the working premise of flash-activated experiments [1–5, 9–13] that the usual concentrations (~ 10 μM) of added redox mediators are sufficient to equilibrate the cytochromes with the ambient potential on a timescale of 1 or 2 min, and yet do not significantly interfere with kinetic measurements on a useful timescale [10]. This applies not only in the absence of antimycin, but also in its presence when tendencies to quasi-equilibrium or unwanted disequilibrium would be expected to be enhanced. For example the E_m, and the pK on the reduced form, of cytochrome b_{50} can be measured using flash techniques with 10 μM levels of redox mediators in the presence or absence of antimycin [14], or with 100 μM concentrations of mediators using standard dark redox titrations [10,14].

4. In the presence of antimycin, fig.2,3 show two alternative ways to reduce cytochrome b_{50} so that it is transiently out of equilibrium with the ambient E_h. One uses ferricyanide, and is interpreted as proceeding via cytochrome c_2 and Z. This route requires that cytochrome c_2, oxidized by ferricyanide, undergoes reduction at least once. The second uses a single turnover flash, which oxidizes c_2 via the reaction center. Under these conditions c_2 does not go re-reduced in spite of the fact that b_{50} is fully reduced within 1 or 2 ms of the flash. This apparent dichotomy can be explained if we assume that ZH_2, the reductant of ferricytochrome c_2 [3–5] can reduce c_2 only if it can also transmit an electron to b_{50}. If antimycin slowed this rate from a halftime of about 1 ms [4,5] to the 7 ms measured in the presence of antimycin in mitochondria [15] and *Paracoccus denitrificans* [16], then the addition of antimycin could allow the electron ejected from the primary acceptor of the reaction center to reduce cytochrome b_{50} via Q · H before an electron could arrive from ZH_2. In such a case the premature reduction of the cytochrome *b* would prevent the reduction of ferricytochrome c_2 by ZH_2. As before [1], space limitations do not permit more than this brief discussion of the mode of action of antimycin, which will be the subject of a forthcoming paper (W. H. van der Berg, et al., in preparation).

5. The experiments reported here, performed under identical conditions to the measurements of proton uptake [1,2], have not revealed a redox reaction with appropriate kinetics to be linked to H^+_{II}. Neither have we found experimental evidence to suggest that H^+_{II} measured at high potential is an artifact of measurement. If the measured presence and kinetics of H^+_{II} do indeed prove to be correct, then H^+_{II} measured at high potential may reflect an as yet unidentified redox reaction which is antimycin sensitive, or an antimycin sensitive 'Böhr proton' [17]. The high potential H^+_{II} could even be quite different from the H^+_{II} measured with Z reduced at the time of the flash. Clearly more experiments are needed before we will be in a position to design a model that satisfactorily explains all the data.

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