Kinetics of electron transfer in reaction center-cytochrome *o* proteoliposomes

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A proteoliposome hybrid protein system consisting of photosynthetic reaction centers (RC) from *Rhodopseudomonas sphaeroides*, cytochrome *o* from *E. coli* and ubiquinone-8 has been reconstituted to study the mechanism of O_2 reduction and the generation of $\Delta\tilde{\mu}H^+$ in cyt. *o*. Steady-state illumination leads to O_2 uptake and the generation of an interior negative $\Delta\psi$. A rapid flash of light induces a single turnover of each RC, and the resultant pulse of quinol leads to quinonoid inhibitor-sensitive millisecond reduction kinetics and cyanide-sensitive re-oxidation kinetics of cyt *o*. The *b* hemes are similar on a kinetic, redox and α-band spectral basis.

Cytochrome o *Reaction center* *Electron transfer* *Proteoliposome*

1. INTRODUCTION

Cytochrome o (cyt.*o*), one of the membrane-bound terminal electron acceptor complexes of the *Escherichia coli* respiratory chain, functions as a ubiquinol/O_2 oxidoreductase (cf. [1,2] for reviews). The 4-subunit enzyme contains two *b*-type hemes [3] and two molecules of copper [4]. The oxidized minus reduced spectrum of the enzyme at 77 K has α-band peaks at 555 and 562 nm, which may or may not be assignable to different *b* hemes [4–6]. O_2 reduction by cyt.*o* is inhibited by azide [7], cyanide and the quinonoid inhibitors, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) [8].

Although the mechanism of electron transfer is not known, on the basis of steady-state electron transfer experiments with cyt.*o* proteoliposomes and ubiquinol-1 (Q_1) it is clear that the enzyme generates a proton electrochemical gradient across the membrane ($\Delta\tilde{\mu}H^+$). Evidence suggests that the membrane potential ($\Delta\psi$) is generated by vectorial electron transfer through the oxidase, while the pH gradient across the membrane (ΔpH) results from scalar reactions at the membrane surface [8,9].

In an attempt to understand the mechanism of O_2 reduction and the generation of $\Delta\tilde{\mu}H^+$ we have reconstituted a proteoliposome hybrid system consisting of photosynthetic reaction centers (RC) from *Rhodopseudomonas sphaeroides*, cyt.*o* from *E. coli* and ubiquinone-8 (Q_8). Light activation of the RC reduces quinone to quinol, while cyt.*o*, in turn, oxidizes quinol and reduces O_2. $\Delta\tilde{\mu}H^+$ generation is coupled to this sequence of reactions. Moreover, a rapid (8 μs) flash of light induces a single turnover of each RC, and the resultant pulse of quinol leads to millisecond reduction kinetics of cyt.*o*.

2. MATERIALS AND METHODS

2.1. *Protein purification*

Cyt.*o* was purified from *E. coli* GR19N by sequential extractions of membranes with urea and cholate, followed by solubilization with oc-

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tylglucoside and DEAE-Sepharose and Sephacryl S-200 column chromatography [3,8,10]. The final preparation was suspended in 50 mM potassium phosphate (KP_i), pH 7.5, containing 1% octyl-β-D-glucopyranoside (octylglucoside).

Chromatophore membranes were obtained by French press treatment of photosynthetically grown *Rps. sphaeroides* R-26 cells. RC were extracted from the membranes with the detergent lauryldimethylamine oxide (LDAO) and purified by ammonium sulfate precipitation and DEAE-cellulose chromatography [11]. Purified RC were dialyzed against 10 mM Tris, pH 8.0, to remove excess salt and detergent. Stock solutions of horse heart cyt.*c* (Sigma type VI) were dialyzed for 1–2 h against 10 mM Tris, pH 8.0, to remove excess salt.

2.2. *Proteoliposome preparation*

Reconstitution of the vesicular hybrid system was accomplished either by LDAO reduction/sonication or by detergent dilution, each of which gave preparations with similar kinetic properties. In the former procedure, the hybrid system was reconstituted by sonication and reductive destruction of the LDAO in a method modified from that of Pachence et al. [12]. 4 μmol *E. coli* phospholipid and 0.2 μmol Q_8 were dried in a glass vessel under argon. 20 nmol RC in 4 ml of 50 mM KP_i buffer (pH 7.4) was added followed by simultaneous sonication and reduction to a stable ambient redox potential (E_h) of -200 mV with microliter additions of fresh sodium dithionite solution. 2–4 nmol cyt.*o* was then added, and the solution was briefly sonicated before dialysis against KP_i buffer overnight at 5°C. Proteoliposomes were collected by centrifugation and resuspended to the desired concentration.

Alternatively, proteoliposomes were prepared by a modification of the procedure described by Matsushita et al. [3,8,10]. RC (15 nmol) were adjusted to 1.25% octylglucoside and reduced with sodium dithionite. The RC solution was then added to 3 nmol cyt.*o*, 100 mg *E. coli* phospholipid containing 0.3 μmol Q_8 and sufficient octylglucoside to maintain a concentration of 1.25% in 50 mM KP_i buffer (pH 7.4, 10 ml final volume). The mixture was incubated on ice for 15 min, diluted into 370 ml KP_i buffer at room temperature and stirred for 10 min. The proteoliposomes were collected by centrifugation at 120000 × *g* for 3 h, resuspended in KP_i buffer at a protein concentration of 270 μg/ml and stored in liquid nitrogen. Before use, thawed samples were sonicated for 10–20 s in a bath-type sonicator.

2.3. *Hybrid system activation*

Oxygen uptake was measured with an oxygen electrode at 25°C. The reaction mixture (1 ml final volume) contained proteoliposomes and electron donors in 50 mM KP_i buffer (pH 7.5). Illumination was provided by a slide projector (Kodak Carousel, custom 860H) with full power at a distance of approx. 10 cm from the electrode. Illumination causes an unexplained immediate increase of the oxygen electrode voltage (fig.1) even in the absence of enzyme.

The $\Delta\psi$ was determined by measuring the steady-state distribution of [^{3}H]tetraphenylphosphonium (TPP, bromide salt) by flow dialysis [13].

The kinetics of RC and cyt.*o* electron transfer in the proteoliposomes were observed with a double-beam kinetics spectrophotometer (Johnson Research Foundation). The sample was kept under argon in an anaerobic glass cuvette fitted with a calomel-platinum electrode pair. Redox mediator dyes were used at minimal concentrations needed to establish effective redox potential readings on a tens of seconds time scale and prevent interference with millisecond electron transfer kinetics [14]. Excitation of the RC was accomplished with a xenon flash of approx. 8 μs passing through a Wratten 88A infrared filter. Data were accumulated using a Biomation waveform recorder interfaced with an IBM personal computer.

3. RESULTS

The construction of an RC hybrid protein system permits the light-regulated production of ubiquinol. It has been shown in previous studies of the light-initiated electron transfer reactions of the RC/mitochondrial quinol-cyt.*c* oxidoreductase (bc_1) hybrid system [15] that a photon absorbed by the RC stimulates a charge separation resulting in the reduction of ubiquinone to ubiquinol and the oxidation of bacteriochlorophyll dimer $(BChl)_2$. The oxidized $(BChl)_2$ is subsequently restored to its initial state upon rereduction by cyt.*c* and ascorbate or by ferrocene. The light-generated ubi-

quinol then reduces cyt.*b* and cyt.c_1. In the RC/cyt.*o* hybrid system, the light-generated quinol will be shown to reduce cyt.*o* and lead to reduction of O_2.

3.1. *Oxygen consumption and potential generation*

Discounting the artifactual light-induced increase in O_2 electrode voltage observed in the absence of proteoliposomes, exposure of the RC/cyt.*o* hybrid system to light causes immediate uptake of O_2 (fig.1). Furthermore, O_2 reduction is severely inhibited by HQNO and returns to the low background rate when the light is turned off.

As demonstrated by TPP^+ distribution studies (fig.2, bottom), RC/cyt.*o* proteoliposomes generate a light-induced $\Delta\psi$ of the same polarity (interior negative) as that observed in proteoliposomes containing cyt.*o* alone [3,8]. The $\Delta\psi$ of -100 to -115 mV produced in hybrid proteoliposomes may be due to turnover of RC and cyt.*o*, since an interior negative $\Delta\psi$ is generated by both RC proteoliposomes in the presence of Q_1 (fig.2, top) and by hybrid proteoliposomes supplied with Q_1H_2 without illumination (not shown).

Light-dependent O_2 consumption and $\Delta\psi$ generation are dependent on the molar ratio of RC to cyt.*o* in the proteoliposomes (fig.3). As shown,

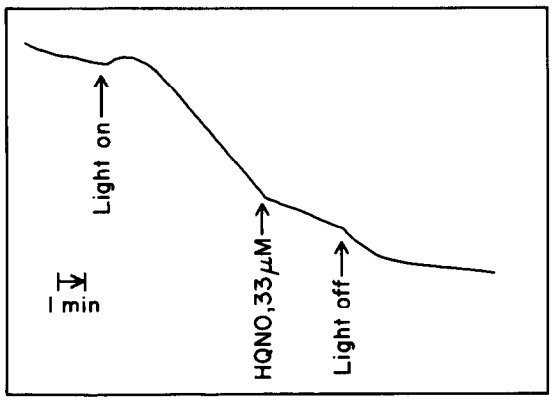

Fig.1. Light-induced oxygen consumption in RC/cyt.*o* hybrid proteoliposomes. Proteoliposome concentrations were 9 nM cyt.*o*, 45 nM RC, 700 μM *E. coli* phospholipids and 3.9 μM Q_8 with electron donors 10 mM ascorbate and 20 μM cyt.*c*, in 50 mM KP_i, pH 7.4. O_2 consumption rates were 4.15 and 0.73 μgatom O/min per mg protein before and after addition of HQNO, respectively.

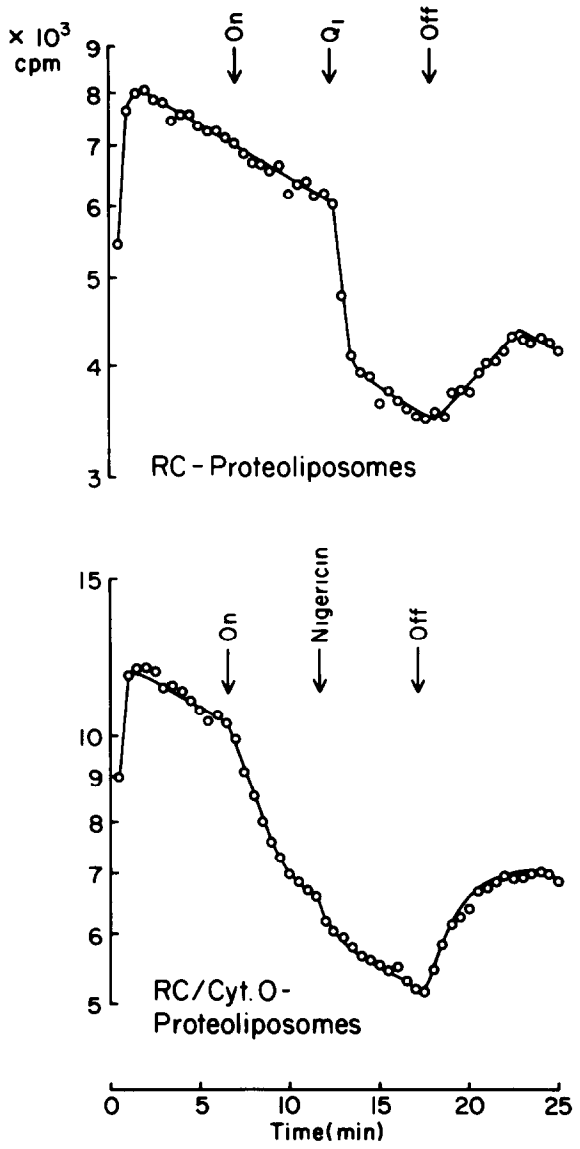

Fig.2. Light-induced transmembrane potential formation in RC or RC/cyt.*o* hybrid proteoliposomes. [^{3}H]TPP distribution measurements were performed by flow dialysis. The 0.2 ml reaction mixture in 50 mM KP_i, pH 7.4, contained either 470 nM RC and 8.3 mM *E. coli* phospholipids (top trace), or 113 nM cyt.*o*, 565 nM RC, 8.75 mM *E. coli* phospholipids and 48.6 μM Q_8 (bottom trace). The experiment was begun after the addition of 20 μM [^{3}H]TPP (2.5 Ci/mmol) and uptake was initiated by the addition of 10 mM ascorbate/20 μM cyt.*c*. Where indicated, 80 μM Q_1 or 0.25 μM nigericin was added.

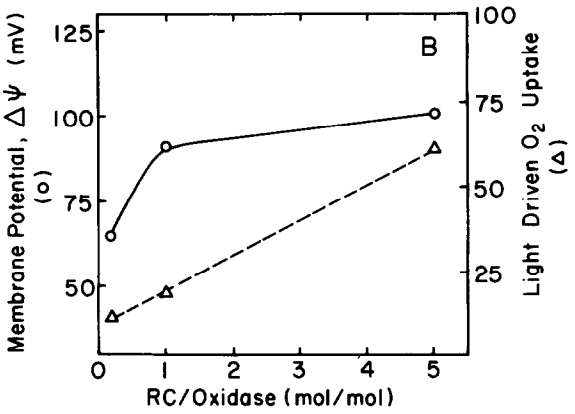

Fig.3. Effect of RC/cyt.*o* molar ratio on light-induced O_2 consumption and $\Delta\psi$ formation in RC/cyt.*o* hybrid proteoliposomes. Hybrid proteoliposomes were prepared with constant cyt.*o* and Q_8 concentration (molar ratio 1:100) but variable RC concentration. Measurement was made as in the legends of figs 1 and 2. O_2 consumption is expressed as the % relative activity compared with that observed in the dark with 5 mM dithiothreitol and 80 μM Q_1 as electron donors.

the rate of O_2 uptake increases essentially linearly over the entire range of RC:cyt.*o* ratios studied. Moreover, the rate of O_2 consumption is further enhanced by increasing the molar ratio of Q_8 to RC up to 50 (not shown). It seems likely, therefore, that generation of quinol by the RC and its diffusion is rate-limiting for O_2 uptake during steady-state continuous illumination. In contrast, the $\Delta\psi$ generated under the same conditions increases from about -65 mV at an RC:cyt.*o* ratio of 0.2 to about -90 mV at unity to about -100 mV when RC is present in 5-fold excess over cyt.*o*. Thus, as shown previously in proteoliposomes reconstituted with the D-lactate oxidase system (K. Matsushita and H.R. Kaback, submitted), relatively low rates of electron transfer are required to generate a maximum $\Delta\psi$.

3.2. *Single-turnover activation*

A major virtue of the hybrid system as an experimental tool is that flash activation initiates a single turnover of the redox centers in the RC [16]. Reducing and oxidizing equivalents can then be delivered to other components of the hybrid system in precisely controlled pulses without the necessity for large pools of oxidizing or reducing substrate, thereby providing a degree of clarity that is impossible to achieve under steady-state electron transfer experiments.

To observe the spectral response of the oxidation of quinol by cyt.*o*, RC/cyt.*o* vesicles were maintained in an anaerobic cuvette at a redox potential of about 230 mV, essentially oxidizing the *b* cytochrome complement. After flash activation of the RC and reduction of Q_8, the oxidized $(BChl)_2$ is rapidly rereduced by ferrocene, an electron donor with a negligible spectral change in the 560 nm region. The absorbance change that results (fig.4) is very similar to the chemically reduced minus oxidized spectrum of purified cyt.*o*, including the characteristic broad α-band (cf. [4,8]).

The extent of *b* heme reduction on a single flash is directly proportional to the amount of $(BChl)_2$ flash oxidized. Multiple flash activation of the RC leads to increased reduction of cyt.*b*, indicating that the concentration of flash-generated Q_8H_2 is limiting in these experiments.

3.3. *Electron transfer kinetics*

The kinetics of *b* heme reduction by Q_8H_2 were studied at wavelength pairs that contain no spectral contribution from $(BChl)_2$ oxidation. In addition, ferrocene was excluded from the solution to clarify the RC spectral isosbestics. The left half of fig.5 shows electron transfer kinetics of *b*-type heme, measured at 563–526 nm, over two time

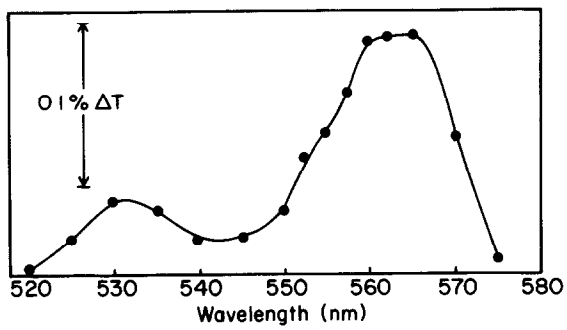

Fig.4. Flash-induced spectral change of RC/cyt.*o* proteoliposomes. The change in transmittance was measured 200 ms after a xenon flash. The reaction mixture contained 2 μM RC, 0.4 μM cyt.*o*, 800 μM *E. coli* phospholipids, 40 μM Q_8, 50 mM KP_i (pH 7.4), 40 μM ferrocene, 5 μM ferro-EDTA, 0.2 μM diaminodurene (DAD), 1 μM 2-hydroxynaphthaquinone (OHNQ); E_h 220 mV.

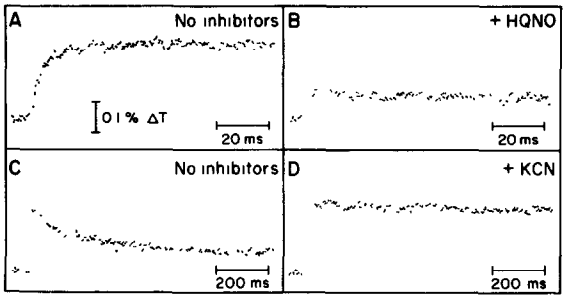

Fig.5. Kinetics of xenon flash-induced cyt.*b* reduction at 563–526 nm in RC/cyt.*o* proteoliposomes. The reaction mixture contained 2 μM RC, 0.8 μM cyt.*o*, 800 μM *E. coli* phospholipids, 40 μM Q_8, 50 mM KP_i (pH 7.4), 2 μM ferro-EDTA, 0.1 μM DAD, pyocyanine; E_h 200 mV. Where indicated, 50 μM HQNO, or 0.2 mM KCN were added.

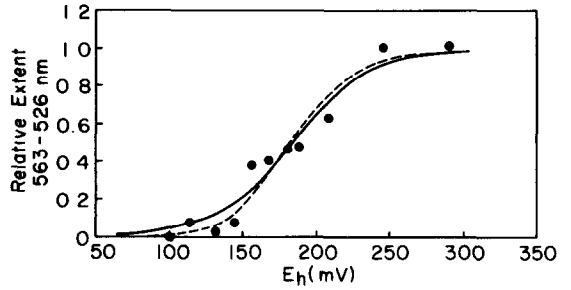

Fig.6. Redox titration of flash-induced transmittance change at 563–526 nm in RC/cyt.*o* proteoliposomes. (——) n = 1 theoretical Nernst curve, midpoint 185 mV. (– – –) Theoretical curve for both *b* hemes in cyt.*o* oxidized, with the 160 mV midpoint for both hemes, given by the relation:

$$[\text{both hemes oxidized}] = [\text{cyt.}o]/(1 + 10^{(E_m - E_h)/59})^2$$

at room temperature where E_h is the redox potential and E_m the redox midpoint. The reaction mixture contained 1 μM RC, 0.2 μM cyt.*o*, 200 μM *E. coli* phospholipids, 10 μM Q_8, 50 mM KP_i, pH 7.4, 20 μM ferro-EDTA, 5 μM DAD, 10 μM OHNQ.

scales. Trace A shows *b* heme reduction taking place with a half-time of 4 ms. Trace C, at 10-fold longer time scale, shows *b* heme reoxidation taking place with trace quantities of introduced oxygen, and as shown in trace D, reoxidation is effectively eliminated by cyanide. Although not shown, cyt.*o* reduction measured at 558 minus 528.5 nm is not significantly different from that observed at 563 minus 526 nm.

The extent of *b* heme reduction is inhibited by about 70% in the presence of HQNO, although the residual rate of reduction is relatively rapid (fig.5B). Another quinone analogue, UHDBT, causes a similar, non-additive, degree of inhibition.

Redox titration of the flash-activated RC/cyt.*o* hybrid system indicates that the extent of *b* heme reduction by ubiquinol at pH 7.4 decreases steeply with redox potential around 185 mV (fig.6). There is no indication of a multiphasic dependence on potential to suggest that there is more than one redox component.

4. DISCUSSION

These experiments demonstrate that RC and cyt.*o* may be co-reconstituted into electrically tight proteoliposomes that maintain high quinol/O_2 oxidoreductase activity and in which a majority of cyt.*o* molecules are oriented to generate an interior negative $\Delta\tilde{\mu}H^+$. At RC to cyt.*o* ratios greater than unity, the light-induced $\Delta\tilde{\mu}H^+$ reaches a limiting value of about -100 mV which reflects H^+ uptake and release and vectorial electron flow by both the RC and cyt.*o* balanced by compensatory ion leaks across the membrane.

The observed flash-stimulated initial rate of *b* heme reduction, when normalized by the total amount of *b* heme reduced, gives a rate of 200 s^{-1} per heme reduced, a value that compares favorably with the turnover number for cyt.*o* in the inner membrane of *E. coli* and in purified enzyme preparations [4,8]. In both the continuous illumination and flash-excited experiments, the overall reaction rate is dependent upon the amount of RC excited. In view of the rapid quinone reduction reaction in the RC (200 μs half-time), it is likely that the diffusion of Q_8H_2 from RC to cyt.*o* dictates the observed rates of *b* heme reduction and O_2 uptake.

The RC/cyt.*o* hybrid system provides a means of performing a kinetic or functional, as opposed to equilibrium, redox titration of cyt.*o*. The ability of *b* heme to become reduced when presented with QH_2 from the RC falls off with decreasing redox potential around 185 mV. The data can be fitted to an n = 1 Nernst curve, suggesting that the equilibrium reduction of an n = 1 redox component, such as *b* heme itself, prevents flash reduc-

tion. In comparison, a dark equilibrium redox titration gives a midpoint of approx. 150 mV (not shown). The discrepancy in midpoints suggests several possibilities that cannot be immediately resolved: the functional midpoint of *b* heme may be 35 mV higher than the equilibrium midpoint; another redox component with a midpoint around 185 mV (perhaps a copper) controls the reduction of *b* heme by QH_2; or the flash-induced reduction of *b* heme might require that both hemes are oxidized before the flash so that the quinol can be doubly oxidized. This type of mechanism, which generates a redox curve that is slightly steeper than $n = 1$, gives a midpoint of the *b* hemes that is 25 mV lower, or 160 mV.

In principle, flash-induced quinol production by the RC could provide a means of functionally distinguishing between the two *b* hemes in cyt.*o*. However, (i) flash reduction of cyt.*o* generates a difference spectrum which retains the same form as the total redox spectrum, (ii) cyt.*o* reduction kinetics at various wavelengths do not exhibit more than one component, and (iii) flash-induced cyt.*o* reduction titrates with monophasic dependence on redox potential. If quinol must reduce both *b* hemes in an $n = 2$ reaction, the hemes are not distinguished in the hybrid system. On the other hand, if quinol reduces only one *b* heme directly, the second heme must have similar redox properties. Moreover, considering the monophasic kinetics of heme reduction, both hemes may be in rapid (submillisecond) redox equilibrium with each other and/or have similar α-band absorptions. These possibilities are consistent with other experiments [17] showing that the two *b* hemes in cyt.*o* are marginally distinguishable on a redox basis and can only be distinguished spectrally in the α-band region on the basis of carbon monoxide perturbation of second or higher order difference spectra [4].

Although the overall data with cyt.*o* proteoliposomes are consistent with the notion that cyt.*o* catalyzes vectorial transfer of electrons but not protons [8,9], the possibility of a transmembrane proton transfer has not been completely eliminated. Further studies of the single-turnover reactions of the oxidase in the RC/cyt.*o* hybrid system may permit resolution of the steps involved in electron transfer, protonation and deprotonation and $\Delta\psi$ generation within the oxidase and lead to a clear mechanism.

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