Biochimica et Biophysica Acta, 766 (1984) 322–333
Elsevier

BBA 41543

A NEW ELECTROGENIC STEP IN THE UBIQUINOL: CYTOCHROME c_2 OXIDOREDUCTASE COMPLEX OF *RHODOPSEUDOMONAS SPHAEROIDES*

ELZBIETA G. GLASER and ANTONY R. CROFTS

Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, 524 Burrill Hall, 407 S. Goodwin Avenue, Urbana, IL 61801 (U.S.A.)

(Received January 19th, 1984)

Key words: Ubiquinol: cytochrome c_2 oxidoreductase; Myxothiazol; Electrogenic process; Antimycin sensitivity; Carotenoid; (Rps. sphaeroides)

Myxothiazol, an inhibitor of the ubiquinol oxidase site of the ubiquinol: cytochrome c_2 oxidoreductase complex, has been shown in the present work to inhibit a part of the electrogenic process indicated by phase III of the carotenoid change, in addition to the part of the change inhibited by antimycin. This finding shows that there is an antimycin-insensitive, but myxothiazol-sensitive portion of the slow phase, which indicates the existence of an electrogenic event within the ubiquinol: cytochrome c_2 oxidoreductase complex, in addition to that linked to oxidation of cytochrome *b*-561 which has been previously characterized. Redox titrations show that the appearance of the new electrogenic step is correlated with the amount of cytochrome *b*-561 available in the oxidized form before the flash. The rate of the antimycin-insensitive and myxothiazol-sensitive portion of the carotenoid change correlates well with the rate of reduction of cytochrome *b*-561. No carotenoid change associated with reduction of cytochrome *b*-566 was seen. These findings suggest that the newly identified electrogenic process is linked to electron transfer between cytochrome *b*-566 and *b*-561. Calculations of the contribution of this new electrogenic step to the total electrogenic event within the complex show that electrons passing from cytochrome *b*-566 to cytochrome *b*-561 pass about 35–50% of the distance across the whole membrane.

Introduction

The ubiquinol: cytochrome c_2 oxidoreductase complex (QH_2-Cyt c_2) of *Rhodopseudomonas sphaeroides* catalyzes cyclic electron transport, accepting electrons from the photochemical reaction center through ubiquinol, and delivering them through cytochrome c_2. Recently, a modified Q-cycle mechanism has been proposed to account for the function of the complex [1]. The mechanism assumes that the complex oxidizes ubiquinol (QH_2) from the pool and reduces cytochrome c_2 in reactions which are kinetically independent of the photochemical reaction center. The modified Q-cycle is developed from earlier Q-cycles [2–5] and, in particular, incorporates the suggestion of Garland et al. [6] for a double-turnover mechanism. The development of this model is discussed extensively elsewhere [1,7]. The ubiquinol oxidation is proposed to occur in a two-step concerted reaction in which one electron is delivered to a high-poten-

Abbreviations: BChl, bacteriochlorophyll; Mops, 3-[*N*-morpholino]propanesulfonic acid; Hepps, *N*-2-hydroxyethylpiperazine-*N'*-2-propanesulfonic acid; P_{860}, P_{860}^{+}, reaction center primary donor, reduced and oxidized forms; Cyt, cytochrome; FeS, Rieske-type iron sulfur center; Q_A, bound ubiquinone, which acts as the primary stable acceptor of the reaction center; Q, QH_2, ubiquinone, ubiquinol; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazol; UHNQ, 3-*n*-undecyl-2-hydroxy-1,4-naphthoquinone.

tial chain, and the second electron enters a chain containing the *b*-cytochromes. The high-potential chain consists of the Rieske type iron-sulfur protein (FeS), cytochrome c_1, cytochrome c_2, and the reaction center (P), and the low-potential chain consists of cytochrome *b*-566, cytochrome *b*-561 and a quinone reductase site [7–19]. The complex has been recently isolated [13,20] and the polypeptide composition has been characterized. However, there is no biochemical evidence available on the topological arrangement of the complex in the membrane except for the location of cytochrome c_2 [21] on the periplasmic side of the membrane (i.e., inside chromatophores). On the basis of kinetic studies, three distinct catalytic sites of the complex have been identified: (a) a ubiquinone-reductase site, (b) a ubiquinol-oxidase site, and (c) the cytochrome c_2 reductase site. The first of these sites has been proposed [1,22,23] to be exposed to the side of negative protonic potential (N-side, i.e., outer side of chromatophores), and the other two on the periplasmic or positive side (P-side) (the Cyt c_2-containing phase, inside the chromatophore). The quinone-reaction sites are assumed to have contact with both the lipid and aqueous phases, to allow equilibration with the quinone pool and protons [1,22,23].

The electrochromic absorbance changes of carotenoids and bacteriochlorophyll have been shown to be proportional to membrane potential [24–27], and have been widely used as a measure of electrogenic processes in the membrane. If the stoichiometry of charge transfer involved in the electrogenic process is known, the distance of the electrogenic step in the membrane can be calculated. As a result of such calculations, the locations of the sources and sinks for electrogenic processes can be approximately estimated in the membrane.

The flash-induced carotenoid change in chromatophores consists of three phases [27–29] of which phase I and phase II are referred to as the fast phase, and are associated, respectively, with the photochemical reaction in the reaction center ($P_{860}Q_A \rightleftharpoons P^+_{860}Q^-_A$; $t_{1/2,I} = 200$ ps) and the reduction of P^+ by cytochrome c_2 (Cyt $c_2 + P^+_{860}Q^-_A \rightleftharpoons$ Cyt $c_2^+ + P_{860}Q^-_A$, $t_{1/2,II} = 3$ μs). Phase III is referred to as the slow phase of the carotenoid change and is attributed to electrogenic events involved in electron transfer through the ubiquinol : cytochrome c_2 oxidoreductase complex. The kinetics of the slow phase are dependent on ambient redox potential. The faster kinetics, with $t_{1/2} = 1$–2 ms, appear on reductive titration over the range below 150 mV at pH 7.0. The slower kinetics with $t_{1/2} \approx 10$ ms, are observed over the higher potential range at $E_{h,7} > 160$ mV [1,30–32]. The increase in rate of the slow phase at potentials below 150 mV, previously ascribed to reduction of a bound ubiquinone, Q_z, is now thought to be associated with an increased rate of oxidation of ubiquinol, as the concentration of this substrate increases on reduction of the pool [1]. Antimycin A, which binds to the ubiquinone-reductase site [1] and inhibits cytochrome *b*-561 oxidation [28–31], also inhibits phase III [25–27]. Because of this, it had been concluded that the electrogenic event visualized by Phase III of the carotenoid change was associated with the oxidation of cytochrome *b*-561 and reduction of ubiquinone [1,30–32], as described by the reaction:

$$\text{Cyt } b\text{-}561^- + Q \rightleftharpoons \text{Cyt } b\text{-}561 + Q_C^{\cdot -}$$

$$\text{Cyt } b\text{-}561^- + Q_C^{\cdot -} + 2H^+ \rightleftharpoons \text{Cyt } b\text{-}561 + QH_2.$$

In the present work, we have studied the carotenoic bandshift change in the presence of antimycin and/or myxothiazol. Myxothiazol is an inhibitor of the ubiquinol oxidase site of the ubiquinol : cytochrome c_2 oxidoreductase complex [33–36]. Although the reduction of both the *b*-type cytochromes, and the Rieske type FeS center is inhibited, the oxidation of the FeS center is not. Our data on the effect of myxothiazol on the carotenoid bandshift change show that there is an antimycin-insensitive, but myxothiazol-sensitive portion of phase III. This indicates the existence of an electrogenic event within the ubiquinol : cytochrome c_2 oxidoreductase complex, in addition to that previously characterized as oxidation of cytochrome *b*-561. Studies of the redox characteristics of this process indicate that this electrogenic step exists between cytochromes *b*-566 and *b*-561 and that it contributes 35–50% of the total phase III change.

Materials and Methods

Chromatophores of *Rhodopseudomonas sphaeroides* strain GA were prepared as described previously [37]. The kinetics of flash-induced carotenoid electrochromic changes and cytochrome *b*-561 reduction were measured using a computer-linked kinetic spectrophotometer as described earlier [16]. Redox poising was done by small additions of concentrated solutions of sodium dithionite or potassium ferricyanide. The carotenoid electrochromic change was measured at 503 nm [1] and cytochrome *b*-561 reduction as the difference between kinetics measured at 561 and 569 nm [7].

The reaction center, cytochrome *b*-561, and cytochrome *c* content were measured in order to characterize chromatophores. The reaction center change was estimated at 542 nm, cytochrome *b*-561 reduction as above, and cytochrome *c* oxidation at 551 − 542 nm (Cyt $(c_1 + c_2)$), from the extent of the absorbance change after a group of four or eight flashes in the presence of antimycin A, and under uncoupled conditions (valinomycin, 2 μM; nigericin, 2 μM) at E_h 200 mV. Using these wavelengths, an extinction coefficient of $\varepsilon = 19.5$ $mM^{-1} \cdot cm^{-1}$ was used for both Cyt *b*-561 and for Cyt $(c_1 + c_2)$, and of $\varepsilon = 10.3$ $mM^{-1} \cdot cm^{-1}$ for the reaction center. Flash-induced time-resolved spectra were obtained from kinetic measurements at 24 wavelengths in the range 480–520 nm [38]. Bacteriochlorophyll content was estimated after acetone extraction by a procedure described elsewhere [39].

The reaction mixture normally employed consisted of chromatophores suspended in an anaerobic medium containing 50 mM buffer (Mops (pH 7.0)/Hepps (pH 8.35)) and 100 mM KCl, redox mediators and antimycin A or myxothiazol when indicated. A stream of oxygen-free argon was flowed over the reaction mixture during experiments. In all experiments, the concentration of chromatophores was adjusted so that a single flash induced turnover in more than 90% of the reaction centers. For all experiments, the flash lamp used was EG and G FX201, discharging a 3 μF capacitor charged to 1.5 kV. The flash duration measured in the actinic spectral range was approx. 4 μs at half-maximal amplitude. In all experiments, a dark period of 1 min was allowed between flashes or groups of flashes. The concentration of mediators was varied at different pH values as described in Ref. 7, and is indicated in figure legends.

Valinomycin and antimycin A were obtained from Sigma, and UHNQ from Aldrich. UHDBT was a kind gift from Dr. Trumpower; myxothiazol was a kind gift from Drs. Reichenbach, Thierbach and Trowitzsch, and nigericin was a gift from E. Lilly Pharmaceuticals.

Results

Fig. 1 shows typical traces of the carotenoid electrochromic change induced in dark-adapted chromatophores by a single flash. The change was measured at 503 nm in the presence of antimycin or myxothiazol, or in the absence of the inhibitors, at different values of E_h. The traces at two values of ambient potential are shown, 100 and 200 mV, at pH 7.0 (Fig. 1, A and B). At 100 mV, maximal values of both rate and extent of the slow-phase carotenoid change (phase III) are reached. At 200 mV, the slower phase III can be observed. The combined extent of the fast phases (phases I and II) of the carotenoid change is nearly constant over this potential range. Antimycin inhibits a great part of the slow-phase change at both potentials; however, in both cases, there is an additional portion of the slow phase which is inhibited by myxothiazol. Right hand panels of Fig. 1 show the portion of the total carotenoid change which is sensitive to myxothiazol (C, D) and the portion of the slow phase which is sensitive to myxothiazol, but insensitive to antimycin (E, F). In the presence of a 5-fold excess of myxothiazol over the amount used in the experiment described above, the extent and kinetics of the carotenoid change were the same as those shown in Fig. 1, indicating that myxothiazol had no uncoupling effect. It is worth noting that at 200 mV, the extent of the slow phase has not reached its maximal value during the time measured, i.e., after 16 ms. Addition of UHDBT or UHNQ [8,9], which also inhibit the quinol oxidase site of the complex at low concentration, gave effects similar to those observed using myxothiazol (not shown). However, at higher concentrations, and especially with UHNQ, a par-

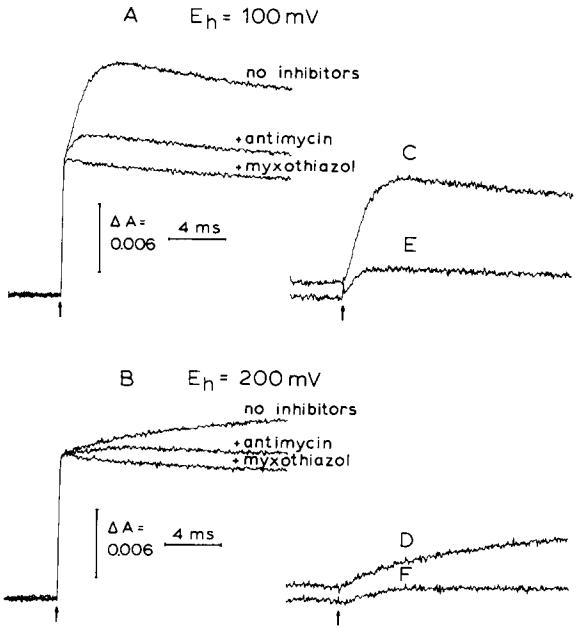

Fig. 1. Kinetics of the carotenoid electrochromic change. Chromatophores were suspended in 50 mM Mops-buffer containing 100 mM KCl (pH 7.0) and redox mediators: phenazine methosulfate, phenazine ethosulfate, pyocyanine at 1 μM, 1,2-naphthoquinone, 1,4-naphthoquinone at 10 μM, and 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD) at 2 μM. Concentration of BChl in the cuvette was 27 μM, (reaction center, 0.24 μM; cytochrome *b*-561, 0.11 μM; cytochrome ($c_1 + c_2$) 0.23 μM); 10 μM antimycin A and 3 μM myxothiazol were present when indicated. Traces A and B (average of four, sweep 20 ms full scale, time constant 20 μs) show the change at 503 nm at 100 mV (A) and 200 mV (B), induced by excitation with one flash; traces C and D show the differences between traces without inhibitor and with myxothiazol at 100 and 200 mV, respectively; traces E and F show the differences between traces with antimycin and with myxothiazol, at 100 and 200 mV, respectively.

tial loss of amplitude of the fast phase was observed. To a small extent, this loss of amplitude of the fast phase was apparent even at the low concentrations required to inhibit turnover of the complex. Since the effect is not yet fully characterized, in all experiments reported here, myxothiazol was used to inhibit the quinol oxidase site of the complex.

In Fig. 2, the extents of the carotenoid electrochromic change in the presence of antimycin or myxothiazol are plotted as a function of redox potential, over the ranges appropriate for measurements at pH values of 7.0 (A, C) and 8.35 (B, D). Figs. 2A and 2B show the maximal extent of the change, which occurred at different times after the flash depending on potential. In the presence of antimycin, the change reached a constant maximal extent over the range of E_h between 240 and 150 mV at pH 7.0 (or between 160 and 70 mV at pH 8.35). Thereafter, a small increase of the maximal extent of the change could be observed, titrating in over the range of E_h between 150 and 80 mV at pH 7.0 (or between 60 and −10 mV at pH 8.35). Below these E_h values, the extent of the change decreased considerably, because of chemical reduction of Q_A before the flash. Corresponding titration curves in the presence of myxothiazol show a maximal extent which was stable over the high potential range, and nearly equal to the change obtained in the presence of antimycin. The values decrease slightly over the middle potential range and finally at values of E_h below 20 mV at pH 7.0 (or below about −20 mV at pH 8.35); the titration curves converge with the titration curves obtained in the presence of antimycin. The feature to note in these titration curves is the extra extent of the slow phase observed in the presence of antimycin, but not when myxothiazol was present. This extra extent titrated in over the range in which the quinone pool was reduced, and titrated out over the range in which cytochrome *b*-561 became reduced before the flash.

The curves presented in the right hand panels of Fig. 2 also show the extent of the carotenoid changes as a function of ambient potential. However, the change is measured at a fixed time (16 ms) after the flash. Both the curves obtained in the presence of antimycin, and those in the presence of myxothiazol, show a small decrease over the potential range between 240 and 70 mV at pH 7.0 (or between 160 and −10 mV at pH 8.35). However, the extent of change in the presence of antimycin was consistently higher than in the presence of myxothiazol. The curves converged at about $E_h = 20$ mV at pH 7.0 (or below $E_h = -20$ mV at pH 8.35). Titration curves obtained when both inhibitors were present in the reaction mixture followed the curves obtained in the presence of myxothiazol alone (not shown).

Summarizing the results presented in Fig. 2, it can be concluded that in both cases, either when the maximal extent of the carotenoid change is

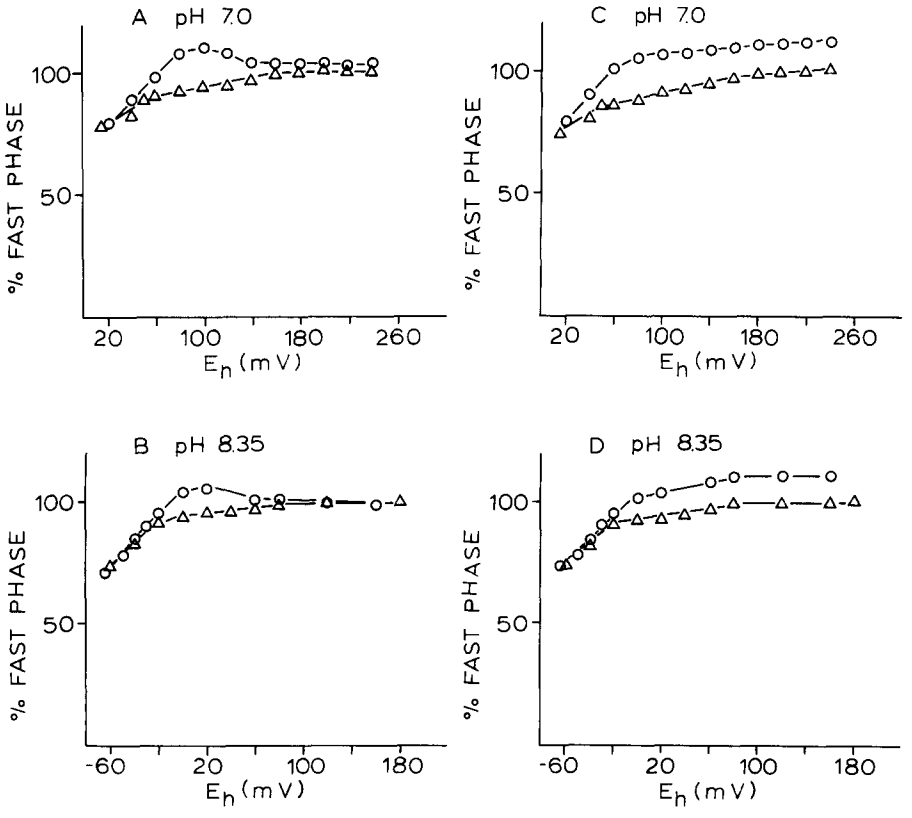

Fig. 2. Redox titrations of extent of the carotenoid electrochromic change. The change was measured at pH 7.0 (A, C), or at pH 8.35 (B, D), at its maximal extent (A, B), or 16 ms after the flash (C, D). The open circles show the change in the presence of antimycin (10 μM), and open triangles show the change in the presence of myxothiazol (3 μM). Chromatophores and the experimental conditions at pH 7.0 were as described in Fig. 1. The reaction mixture at pH 8.35 contained chromatophores (concentrations as in Fig. 1), 50 mM Hepps-buffer, 100 mM KCl, and mediators at concentrations: 0.4 μM each of phenazine methosulfate, phenazine ethosulfate pyocyanin and DAD, and 10 μM each of 1,2-naphthoquinone and 1,4-naphthoquinone. For curves A and B, the value for 100% was taken from the maximal extent of the myxothiazol-insensitive change measured at the highest E_h value shown; for curves C and D, the value for 100% was taken from the extent of the myxothiazol-insensitive change measured 16 ms after the flash, and at the highest E_h value shown.

measured, or the extent 16 ms after the flash, an antimycin-insensitive, myxothiazol-sensitive portion of the slow-phase carotenoid change appears above $E_h = 20$ mV at pH 7.0, or above $E_h = -20$ mV at pH 8.35.

Fig. 3 shows the spectra of flash-induced changes measured at $E_h = 10 \pm 2$ mV and at pH 8.35, over the range 480–520 nm, either in the presence of antimycin alone, or with both antimycin and myxothiazol. The shape of both spectra is characteristic of the carotenoid bandshift change in *R. sphaeroides* GA, with absorption maxima and minima at 503 and 490 nm, respectively. The spectra shown in Fig. 3A and B are measured, respectively, 0.6 and 16 ms after one flash. Myxothiazol is shown to decrease the extent of the carotenoid change in comparison to the change obtained in the presence of only antimycin, at both times measured.

The kinetics of the carotenoid banshift change show a complex set of phenomena, due to different processes in the membrane, as already described in Introduction. In the presence of antimycin, the contribution of the antimycin-sensitive oxidation of cytochrome *b*-561 is eliminated. However, there are at least four kinetic events which contribute to the pattern of the remaining change. The first of these, the change due to the photochemical charge-separation in the reaction center, is readily separated from the slower changes. These

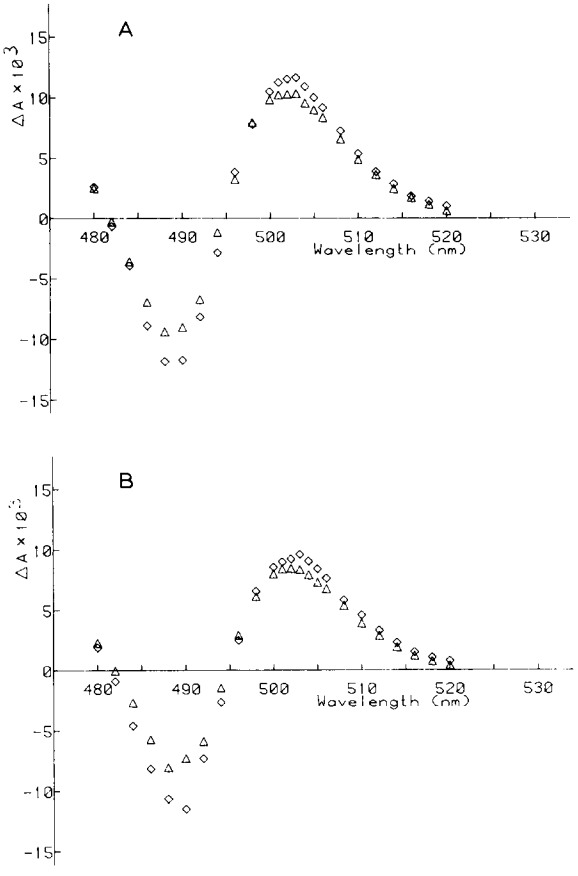

Fig. 3. The flash-induced spectra of the carotenoid bandshift change. The experimental conditions were as in Fig. 2 at pH 8.35. The spectra were measured in the presence of 10 μM antimycin (◇) or in the presence of both antimycin and 3 μM myxothiazol (△), 0.6 ms after the flash (A), or 16 ms after the flash (B). The redox potential was 10 ± 2 mV.

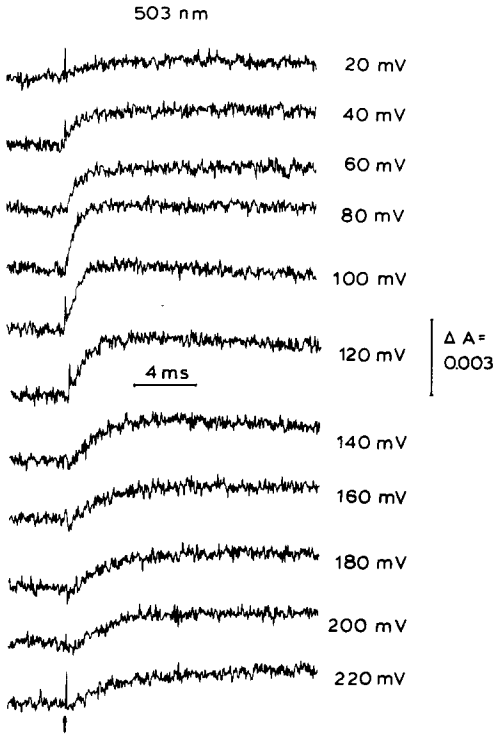

Fig. 4. The antimycin-insensitive and myxothiazol-sensitive portion of the slow phase carotenoid change at different values of ambient redox potential. The experimental conditions for the measurement of carotenoid electrochromic change were as in Fig. 1. Traces obtained in the presence of myxothiazol were subtracted from the traces obtained in the presence of antimycin.

are, reduction of P^+ by cytochrome c_2, reduction of cytochrome *b*-561 by ubiquinol (cf. Figs. 1 and 2) and the passive decay of the membrane potential by leakage of charges through the membrane. In order to dissect these complex kinetic events, we show in the next two figures kinetic traces in which the contributions of particular processes are highlighted by subtraction of the contribution due to previously identified electrogenic events. If the carotenoid bandshift change obtained in the presence of myxothiazol is subtracted from the change obtained in the presence of antimycin, an antimycin-insensitive, but myxothiazol-sensitive part of the slow phase carotenoid change is given. Those differences at several values of E_h are shown in Fig. 4. Two main effects should be noted. Even at potentials of 200 mV and above, a substantial antimycin-insensitive change occurred, with a rise time ($t_{1/2}$) of approx. 7 ms or longer (the value was somewhat variable between preparations, cf. Fig. 1), and an extent which approached approx. 60–70% of the change seen at lower potentials. As the ambient E_h was lowered, the half-time for the rise of the antimycin-insensitive slow phase decreased, with the change in rate titrating in as the quinone pool became reduced before the flash. At E_h values below 80 mV, the extent of the extra slow phase decreased, losing half its amplitude at approx. 50 mV, the $E_{m,7}$ value for Cyt *b*-561.

In Fig. 5, the redox-titration curves of the carotenoid change in the presence of antimycin at 200 mV, pH 7.0 were subtracted from the redox titration curves at lower potentials. At 200 mV, the

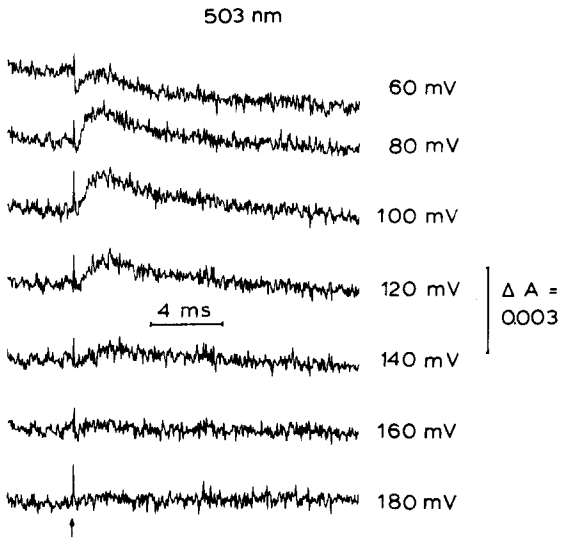

Fig. 5. The effect of E_h on the carotenoid electrochromic change in the presence of antimycin. The experimental conditions were as in Fig. 1. The traces are the difference between the trace at the E_h value shown, and the trace at 200 mV.

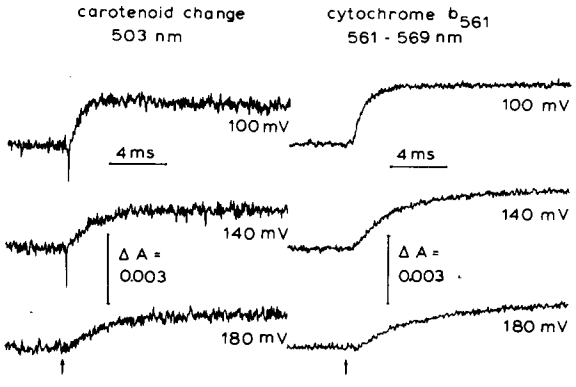

Fig. 6. The antimycin-insensitive and myxothiazol-sensitive portion of the slow phase carotenoid change and reduction of cytochrome *b*-561 at different values of ambient redox potential. Concentration of BChl in the cuvette was 0.25 mM (reaction center, 0.29 μM; cytochrome *b*-561, 0.16 μM; cytochrome ($c_1 + c_2$), 0.27 μM). The carotenoid change was measured as an average of eight, at sweep 20 ms full scale and time constant 20 μs. Other experimental conditions for the measurement of the carotenoid change were as in Fig. 1. Traces obtained in the presence of myxothiazol were subtracted from the traces obtained in the presence of antimycin. The reduction of cytochrome *b*-561 was measured as the difference at 561 − 569 nm in the same reaction mixture as the carotenoid change in the presence of antimycin. Traces were an average of 16, sweep 20 ms, time constant 20 μs. The redox potentials were as indicated in the figure.

high-potential components of the chain are all reduced before the flash, but the ubiquinol pool and the *b*-cytochromes are completely oxidized. The kinetics of the subtracted traces, therefore, reveal effects due to reduction of the pool (over the range below 150 mV), and reduction of the *b*-type cytochromes. Over the potential range below 160 mV, a small transient increase of the change in the initial part of the trace (within 4 ms of the flash) titrated in, with maximal extent and initial rate which increased as the pool became reduced. The transient changes, apparent in the difference kinetics, show that the rate of the antimycin-insensitive slow phase increased, but the final extent was fairly constant, over the E_h range in which the pool became reduced before the flash. At 60 mV, the initial difference became negative, probably due to decreasing amounts of the primary acceptor, Q_A, available in oxidized form before the flash, and the extra slow phase had a smaller maximal extent. This latter effect coincided with the partial reduction of Cyt *b*-561 before the flash.

Since antimycin inhibits the ubiquinone reductase site and myxothiazol inhibits the ubiquinol oxidase site of the complex, the difference traces shown in Fig. 4 should represent the step between these sites involving reductoin of *b*-type cytochromes. In Fig. 6, the kinetics of cytochrome *b*-561 reduction, and of the antimycin-insensitive slow phase are compared at three values of E_h. It can be concluded that the kinetic profiles of the antimycin-insensitive slow phase and of the cytochrome *b*-561 reduction correlate well. This would indicate that the electrogenic process involves reduction of the cytochrome *b*-561. The initial rates of the full myxothiazol-sensitive, and of the antimycin-insensitive but myxothiazol-sensitive slow phases as a function of an ambient potential are shown in Fig. 7. The rate of the antimycin-insensitive slow phase increased about 6-fold over the potential range 160–80 mV. This increase was due mainly to the more rapid rise of the change rather than to a change in extent (as seen kinetically in Figs. 4 and 5), and the half-time decreased accordingly. Below 80 mV, the rate decreased, but this was due mainly to a decrease in the extent of the change, and the half-time re-

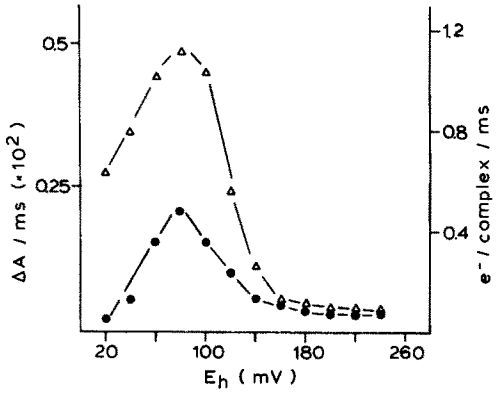

Fig. 7. Rates of the full myxothiazol-sensitive, and of the actimycin-insensitive, myxothiazol-sensitive carotenoid changes as a function of redox potential. Experimental conditions were as in Fig. 1. Open triangles, traces of the myxothiazol-insensitive carotenoid change were subtracted from traces of the uninhibited change, and the initial rate of the difference trace was measured. Closed circles: the traces of the carotenoid change in the presence of myxothiazol were subtracted from the traces in the presence of antimycin and the initial rate of the difference was measured. The rates were expressed in absorbance units/ms (left-hand scale), or as the apparent rate of electron flow through the full electrogenic span of the complex (right-hand scale) (see text). At the maximal extent of the myxothiazol-sensitive carotenoid change at 100 mV, 2 electrons were assumed to pass through the electrogenic process of the complex (see text).

mained fairly constant (see Fig. 4).

The slow phase was further characterized by calculating the extents of the separate contributions to the slow phase measured at a fixed time

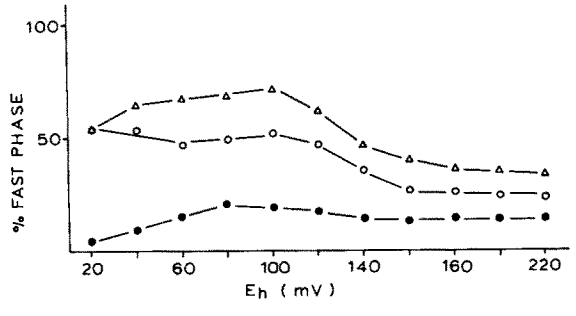

Fig. 8. The dependence on redox potential of the partial reactions of the slow phase. The extents of the myxothiazol-sensitive (△), antimycin-sensitive (○) and antimycin-insensitive but myxothiazol-sensitive (●) carotenoid electrochromic change are plotted as a function of ambient potential. Experimental conditions were as described in Fig. 1. Extent of 100% represents the carotenoid change in the presence of myxothiazol measured at 200 mV, 16 ms after the flash.

(16 ms) after the flash as a function of ambient potential. The values are expressed as a percentage of the myxothiazol-insensitive phase at $E_h = 200$ mV, also measured 16 ms after the flash, and are summarized in Fig. 8. The extent of the total myxothiazol-sensitive phase of the carotenoid change varied from about 35% in the range 240–180 mV, to 72% at 120 mV. The antimycin-sensitive part followed a pattern similar to that of the myxothiazol-sensitive part, and constituted about 25% (at higher E_h) to 53% (at $E_h = 100$ mV) of the myxothiazol-insensitive phase. The portion of the slow phase which was antimycin-insensitive, but myxothiazol-sensitive was constant over the E_h range between 240 and 140 mV at a value of 13%, and increased to 20% at $E_h = 80$ mV. Below 80 mV, the extra change decreased, and disappeared below 20 mV.

Discussion

The mechanism of the ubiquinol : cytochrome c_2 oxidoreductase complex [1] has been already briefly discussed in Introduction. Further discussion of the modified Q-cycle model is necessary in order to interpret the effects of antimycin and myxothiazol on the carotenoid bandshift change. It has been suggested that separate sites of the complex operate in order to oxidize ubiquinol and reduce ubiquinone from the pool [1]. The ubiquinol oxidase site has to turn over twice and the ubiquinone reductase site once, to give a net oxidation of one QH_2 per complex, since two QH_2 must be oxidized at the ubiquinol oxidase site to transfer the two electrons through *b*-cytochrome chain which are required for the reduction of one Q at the ubiquinone reductase site. At E_h values around 200 mV at pH 7.0, the high-potential chain of the complex is completely reduced and the low-potential chain and Q-pool are completely oxidized before the flash. A single flash delivers only approx. 1 QH_2 per complex (per two reaction centers), diffusion of which to the complex is a rate-limiting step for the operation of the ubiquinol oxidase site. At lower ambient potential values (below 150 mV at pH 7.0), the Q-pool is partially reduced before the flash. As a consequence, additional equivalents of QH_2 per complex are available in the pool following the flash, allowing the

complex to turn over more rapidly, and the diffusion of QH_2 from the reaction center to its binding site is no longer a rate-limiting step. The kinetics of these effects can be easily measured in the presence of antimycin, by following the kinetics of cytochrome *b*-561 reduction [1]. In the presence of antimycin, which inhibits the quinone reductase site, the turnover of the quinol oxidase site is restricted by the availability of only two equivalents of acceptor in the *b*-cytochrome chain [1,7]. A further restriction, due to the small value of the equilibrium constant for the reaction by which Cyt *b*-566 is reduced [7], determines that in the presence of antimycin, after a single flash, the quinol oxidase site turns over little more than once, with the low-potential electron from the first turnover reducing cytochrome *b*-561, and from the second partially reducing cytochrome *b*-566. The turnover of the quinol oxidase site in the absence of antimycin can be assayed by measuring the extent of the carotenoid change [1,31]. The extent of the slow phase of the carotenoid change has been found to be twice as great over the potential range around 100 mV, as it is in the higher potential range, above 160 mV. This fact can be explained on the basis of the limited amount of QH_2 available in the pool. At high potentials, the ubiquinol oxidase site turns over rapidly ($t_{1/2} \approx 10$ ms) only once per flash (since only one equiv. of QH_2/ complex is available from the reaction center), transferring only one electron to the quinone reductase site, thus catalyzing only one rapid (approx. 10 ms) electrogenic event per complex. The full extent of the rapid change ($t_{1/2} \approx 1$–2 ms) observed at lower E_h where the amount of QH_2 is no longer a limiting factor, represents two electrons transferred per complex per one flash, as was discussed elsewhere [1]. Antimycin was found to inhibit the slow phase carotenoid change [1,31] both at high and low potentials.

The main point of our work described in the present paper is that there is an electrogenic step in the ubiquinol : cytochrome c_2 oxidoreductase complex in addition to that inhibited by antimycin. This step is inhibited by myxothiazol over a wide range of potentials (cf. Figs. 1, 8, 10 and 13). On the basis of our results, we can answer some interesting questions: (1) where is this step located in the complex? (2) How great is the electrical distance between the reductant and the oxidant involved in this step across the membrane?

The location of the new electrogenic step

Redox titration curves of the carotenoid change (cf. Fig. 2) in the presence of the inhibitors show that when either the maximal extent or the extent 16 ms after the flash are measured, there is a part of the carotenoid change which can be inhibited by myxothiazol, but not by antimycin. The maximal extent measured in the presence of antimycin occurred at different times after the flash (about 5 ms at 200 mV and 1–2 ms at 100 mV). The maximal extent in the presence of myxothiazol occurred (at all potentials) directly (approx. 250 μs) after the flash. In all cases, the kinetics after the flash contain an additional decay component due to leakage of charge across the membrane. The overlapping of these different components accounts for the fact that the curves in Fig. 2A and B coincide at high potentials. If the extent of change was measured at any fixed time after the flash, the myxothiazol-insensitive change was nearly constant over the potential range from 240 to about 80 mV, pH 7.0. The myxothiazol-sensitive, antimycin-insensitive phase began to decrease below 100 mV, and disappeared at approx. 20 mV. In the same potential range, cytochrome *b*-561 starts to be chemically reduced before the flash; with $E_{m,7} = 50$ mV, about 70% of the cytochrome would be reduced at 20 mV. Cytochrome *b*-566 ($E_{m,7} = -90$ mV) is completely oxidized before the flash at 20 mV, and can be largely reduced on illumination by a single flash at this potential [7]. At pH 8.35, the E_m values for the two *b*-type cytochromes are more widely separated on the redox scale, making it easier to measure points of appearance and disappearance of the change. At this pH, $E_{m,8.35}$ for Cyt *b*-561 is 20, whereas $E_{m,8.35}$ for the Q-pool is zero and for Cyt *b*-566 is -180 mV [40]. Kinetic traces at pH 8.35 show that the antimycin-insensitive phase has largely disappeared at $E_h = -20$ mV, where only about 20% of cytochrome *b*-561 is available in the oxidized form before the flash. These results suggest that the electrogenic step measured as the antimycin-insensitive but myxothiazol-sensitive phase of carotenoid change is due to the reduction

of cytochrome *b*-561 and is occurring between cytochrome *b*-566 and cytochrome *b*-561. It seems unlikely that the reduction of *b*-566 represents an electrogenic process, since under the conditions when cytochrome *b*-566 can still be reduced on the flash (below −20 mV, but above the point at which Q_A ($E_{m,8.35} = -81$ mV) would be reduced before the flash), no extra electrogenic process could be detected. Under all conditions tested, the extent of the antimycin-insensitive slow phase reflected the extent of flash-induced reduction of Cyt *b*-561, and, in terms of the mechanisms previously proposed [1], the data could be modelled using a computer program which calculates the changes in concentrations of the components of the chain to be expected shortly after the flash [7]. The extent of the flash-induced reduction of cytochrome *b*-561 in the presence of antimycin, either measured experimentally [41] or calculated theoretically by the computer [7], showed a characteristic small increase over the potential range between 150 and 80 mV, pH 7.0, similar to that observed in Figs. 2, 4, 5 and 8 for the extra carotenoid change in the presence of antimycin, followed by a decrease centered at $E_h \sim 50$ mV as Cyt *b*-561 became reduced before the flash.

The contribution of the new step to the full electrogenic span

The rate of the extra electrogenic step at any value of E_h can be measured from the initial rate of the differences trace obtained by subtraction of the trace in the presence of myxothiazol from the trace in the presence of antimycin. This measured rate can then be compared with the rate of the full myxothiazol-sensitive slow phase, to show the fraction of that rate attributable to the electrogenic step between the two *b*-cytochromes. Such measurements gave values between 0.35 and 0.52 over the range of potentials between 60 and 140 mV, where both changes are maximal (Fig. 7). The rate of the electrogenic process can also be compared with the rate of cytochrome *b*-561 reduction. However, a direct comparison requires a knowledge of the relative electrical distance across the membrane contributed by the span, since the field generated will be a function of the distance through which the charge is moved across the membrane. This distance can be estimated either from the comparison of initial rates above, or from the extents of the changes when these are normalized to account for the number of electrons involved.

The extent of the inhibitor sensitive portion can be expressed as a percentage of the fast phase of the carotenoid change due to charge separation in the reaction center [26–29,41]. In the presence of myxothiazol and antimycin, all reactions of the b-c_1 complex with the quinone pool are inhibited, and the remaining carotenoid change can be attributed to the charge separation in the reaction center and subsequent leakage across the membrane. In order to partly compensate for this latter effect, we have compared the slow phase and its constituent components to the myxothiazol-insensitive carotenoid change measured at the same time after the flash. The extents of both myxothiazol-sensitive and antimycin-sensitive parts of the carotenoid change are in a fairly constant proportion over a wide range of potentials, and in both cases, the extent is twice as great at low potentials as at high potentials, due to transfer of two and one electron, respectively, through the complex [1]. The extent of the myxothiazol-sensitive part of the change constitutes, at low potentials, approx. 70% of the extent of the myxothiazol-insensitive phase measured at 200 mV, and approx. 35% at higher potentials ($E_h > 160$ mV); corresponding values for the antimycin-sensitive change are approx. 50 and approx. 25%. These values are underestimated due to two facts: (1) the extent of the myxothiazol-insensitive phase of the carotenoid change diminished as the potential was lowered (cf. Fig. 2) under our experimental conditions; in part this was due to reduction of Q_A (10% loss at 60 mV), and in part due to a small loss of activity during the course of the titration; (2) the passive decay due to leakage across the membrane is more rapid when the membrane potential is higher. Appropriate correction for these effects would increase the values given above by about 20%. This is consistent with the conclusion that the transfer of two electrons, through the low-potential chain of the complex, creates a membrane potential which is nearly equal to the membrane potential created by charge separation in the reaction center. The antimycin-insensitive, but myxothiazol-sensitive portion of the change constitutes about 13% of

the myxothiazol-insensitive change at high potentials and about 17–20% at low potentials, or approx. 40 and 25–30%, respectively, of the total myxothiazol-sensitive changes at the same ambient potential. These relatively small contributions may account for the fact that the characteristics of this phase have not been noted before. However, as discussed at length above, the change linked to reduction of Cyt *b*-561 measured in the presence of antimycin represents a 1-electron/complex process, whereas the change in the absence of inhibitors is a 2-electron/complex process at $E_h \approx 100$ mV, and a 1-electron/complex process at $E_h > 160$ mV. The amplitude of the antimycin-insensitive, myxothiazol-sensitive change in the E_h range around 100 mV must, therefore, be multiplied by a factor of about 2 in order to estimate the contribution of charge separation through this step to the total electrical work across the membrane, as indicated by the full extent of the slow phase. This leads to values of approx. 50%, similar to the values estimated above from the relative initial rates.

The kinetics of the electrogenic steps

With the above values in mind, we can now compare the rates of the electrogenic event and Cyt *b*-561 reduction shown in Fig. 6. By appropriate normalization of the carotenoid change [1], the initial rate of the overall (myxothiazol-sensitive) electrogenic process of the complex in the absence of inhibitors at $E_h \approx 100$ mV can be shown to be approx. 0.75–1.0 e^-/complex per ms. The apparent initial rate of the antimycin-insensitive, myxothiazol-sensitive change is approx. 0.27–0.36 e^-/complex per ms. The range of values given depends on the choice of normalization procedure. The smaller values are obtained if it is assumed that the extent of the fast phase represents 2 electrons/complex passing through the electrogenic process on each flash; the larger values if the maximal extent of the myxothiazol-sensitive change is used. These values compare with an initial rate of reduction of Cyt *b*-561 in the presence of antimycin of approx. 0.75–0.9 Cyt *b*/complex per ms. Again, the range of values depends on the chocie of normalization procedure. The smaller values are obtained if it is assumed that the full amount of Cyt *b*-561 reduced by four flashes at 200 mV in the presence of antimycin represents 1 Cyt *b*-561/complex; the larger values if the maximal extent of Cyt *b*-561 reduction after one flash at 100 mV in the presence of antimycin is taken to represent the delivery of 1 electron/complex.

In the absence of antimycin, the extent of reduction of Cyt *b*-561 observed kinetically at $E_h \approx 100$ mV is small [7,22,26,41], indicating that the rate of oxidation is almost as fast as the rate of reduction. Under these conditions, both electrogenic processes of the complex (reduction and oxidation of Cyt *b*-561) will be occurring at similar rates, but their contribution to the rate of charging of the membrane (as indicated by the rate of appearance of the electrochromic change) will be determined by the fractional distance across the membrane which each process contributes to the full electrogenic step. The relative rates shown above are, therefore, consistent with the estimate that the reduction of Cyt *b*-561 accounts for approx. 35–50% of the electrical distance across the membrane, and oxidation for the remaining 50–65%.

Summarizing the findings discussed above it can be concluded that the transfer of an electron between cytochrome *b*-566 and cytochrome *b*-561 represents an electrogenic step in the complex, and that the electrical distance electrons have to pass across the membrane, corresponds to about 35–50% of the electrical distance across the whole membrane. These values are in close agreement with estimates for the similar distances in the mitochondrial ubiquonol : cytochrome *c* oxidoreductase complex (30–40%) made by Gopher and Gutman [42], and suggest that the bacterial and mitochondrial complexes have a similar spatial organization in the dimension vertical to the plane of the membrane. The estimate of the fractional contribution to the electrogenic process of electron transfer between the two *b*-cytochromes is also in good agreement with the estimate of Konstantinov et al. [43]. However, these authors concluded that the heme of Cyt *b*-562 (equivalent to Cyt *b*-561 in chromatophores) was electrically close to the M-phase (equivalent to the quinone reductase site), and that Cyt *b*-566 was in the middle of the membrane. Such a distribution would not be consistent with our results.

Acknowledgements

We would like to thank Drs. M. Snozzi and S.W. Meinhardt for useful discussions and their help during this work. We thank also Ms. M.K. Hadden for excellent technical assistance. This research was supported by a grant from the National Institute of Health PHS R01 GM26305. E.G. was supported by an EBMO fellowship.

References

1 Crofts, A.R., Meinhardt, S.W., Jones, K.R. and Snozzi, M. (1983) Biochim. Biophys. Acta 723, 202–218
2 Mitchell, P. (1975) FEBS Lett. 56, 1–6
3 Mitchell, P. (1976) J. Theor. Biol. 62, 327–367
4 Bowyer, J.R. and Trumpower, B.L. (1981) in Chemiosmotic Proton Circuits in Biological Membranes (Skulachev, V.P. and Hinkle, P., eds.), pp. 105–122, Addison-Wesley, Reading, MA
5 Van Ark, G., Raap, A.K., Berden, J.A. and Slater, E.C. (1981) Biochim. Biophys. Acta 637, 34–42
6 Garland, P.B., Clegg, R.A., Boxer, D., Downie, J.A. and Haddock, B.A. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E.C. and Siliprandi, N., eds.), pp. 351–358, North-Holland, Amsterdam
7 Meinhardt, S.W. and Crofts, A.R. (1983) Biochim. Biophys. Acta 723, 219–230
8 Bowyer, J.R., Dutton, P.L., Prince, R.C. and Crofts, A.R. (1980) Biochim. Biophys. Acta 592, 445–460
9 Matsuura, K., Bowyer, J.R., Ohnishi, T. and Dutton, P.L. (1982) J. Biol. Chem. 258, 1571–1579
10 Prince, R.C., Lindsay, J.G. and Dutton, P.L. (1975) FEBS Lett. 51, 108–111
11 Wood, P.M. (1980) Biochem. 189, 385–391
12 Crofts, A.R., Meinhardt, S.W. and Bowyer, J.R. (1982) in Functions of Quinones in Energy Conserving Systems (Trumpower, B.L., ed.), pp. 477–498, Academic Press, New York
13 Gabellini, N., Bowyer, J.R., Hurt, E., Melandri, B.A. and Hauska, G. (1982) Eur. J. Biochem. 126, 105–110
14 Meinhardt, S.W. and Crofts, A.R. (1982) FEBS Lett. 149, 223–227
15 Wood, P.M. (1981) Biochem. J. 192, 761–764
16 Bowyer, J.R., Meinhardt, S.W., Tierney, G.V. and Crofts, A.R. (1981) Biochim. Biophys. Acta 635, 167–186
17 Dutton, P.L. and Jackson, J.B. (1972) Eur. J. Biochem. 30, 495–510
18 Feher, G. and Okamuura, M.Y. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 249–386, Plenum Press, New York
19 Gabellini, N. and Hauska, G. (1983) FEBS Lett. 153, 146–150
20 Yu, L. and Yu, C.-A. (1982) Biochem. Biophys. Res. Commun. 108, 1285–1292
21 Crofts, A.R. (1974) in Perspectives in Membrane Biology, (Estrada, O.S. and Gitler, C., eds.), pp. 373–412, Academic Press, New York
22 Crofts, A.R. and Wraight, C.A. (1983) Biochim. Biophys. Acta 726, 149–185
23 Crofts, A.R. (1983) in The Enzymes of Biological Membranes, 2nd edn. (Martonosi, A.N., ed.), pp. 347–382, Plenum Publ. Corp., New York
24 Jackson, J.B. and Crofts, A.R. (1969) FEBS Lett. 4, 185–189
25 Jackson, J.B. and Crofts, A.R. (1971) Eur. J. Biochem. 18, 120–130
26 Prince, R.C. and Dutton, P.L. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 439–453, Plenum Press, New York
27 Wraight, C.A., Cogdell, R.J. and Chance, B. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 471–511, Plenum Press, New York
28 Dutton, P.L. and Prince, R.C. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 525–570, Plenum Press, New York
29 Crofts, A.R. and Wood, P.M. (1978) Curr. Top. Bioenerg. 1, 175–244
30 Dutton, P.L. and Jackson, J.B. (1972) Eur. J. Biochem. 30, 495–510
31 Prince, R.C. and Dutton, P.L. (1977) Biochim. Biophys. Acta 462, 731–747
32 De Grooth, B.G., Van Grondelle, R., Romijn, J.C. and Pulles, M.P.J. (1978) Biochim. Biophys. Acta 503, 480–490
33 Becker, W.F., Von Jagow, G., Anke, T. and Steplich, W. (1981) FEBS Lett. 132, 329–333
34 Von Jagow, G. and Engle, W.D. (1981) FEBS Lett. 136, 19–24
35 Thierbach, G. and Reichenbach, H. (1981) Biochim. Biophys. Acta 638, 282–289
36 Meinhardt, S.W. and Crofts, A.R. (1982) FEBS Lett. 149, 217–222
37 Bowyer, J.R., Tierney, G.V. and Crofts, A.R. (1979) FEBS Lett. 101, 201–206
38 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536–556
39 Clayton, R.K. (1963) in Bacterial Photosynthesis (Gest, H., San Pietro, A. and Vernon, L.P., eds.), p. 498, Antioch Press, Yellow Springs
40 Prince, R.C., O'Keefe, D.P. and Dutton, P.L. (1982) in Electron Transport and Photophosphorylation (Barber, J., ed.), pp. 197–248, Elsevier Biomedical Press
41 Bowyer, J.R. and Crofts, A.R. (1981) Biochim. Biophys. Acta 636, 218–233
42 Gopher, A. and Gutman, M. (1982) in Functions of Quinones in Energy Conserving Systems (Trumpower, B.L., ed.), pp. 511–526, Academic Press, New York
43 Konstantinov, A., Kunz, W.S. and Kamensky, Y.A. (1981) in Chemiosmotic Proton Circuits in Biological Membranes (Skulachev, V.P. and Hinkle, P.C., eds.), pp. 123–146, Addison-Wesley, Reading, MA