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The effect of the size of the quinone pool on the electrogenic reactions in the ubiquinol-cytochrome c_2 oxidoreductase of *Rhodobacter capsulatus*. Pool behaviour at the quinone reductase site

Giovanni Venturoli [a], Javier G. Fernández-Velasco [a,*], Antony R. Crofts [b] and B. Andrea Melandri [a]

[a] *Dipartimento di Biologia, Istituto ed Orto Botanico, Università di Bologna, Bologna (Italy)* and [b] *Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, Urbana, IL (U.S.A.)*

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(i) Electron transfer within the ubiquinol-cytochrome c_2 oxidoreductase (bc_1) complex produces a charge separation across the membrane which, in some bacterial chromatophores, can be monitored by the electrochromic band shift of carotenoids. The electrogenic steps are believed to coincide with the electron transfer through the cytochrome *b* chain to the quinone reductase site (Q_C) of the complex. (2) The kinetics of these electrogenic reactions have been studied in chromatophores from *Rhodobacter capsulatus*, activated by a single turnover flash, as a function of the ambient redox potential, and of the size of the ubiquinone pool in isooctane-extracted membranes. (3) In chromatophores containing the native ubiquinone (UQ) pool the rate of charge transfer, monitored by the electrochromic red shift of carotenoids, is progressively increased by lowering the E_h below 160 mV. At 100 mV the rate, when normalized to the number of reaction centers turning over, reaches a maximum and declines significantly when the E_h is lowered further (down to 40 mV). This rate remains constant for E_h values between 40 mV and −40 mV. (4) When the size of the UQ pool is decreased by isooctane extraction, the stimulation in the rate of charge separation takes place only at more negative E_h values and the value of the maximum rate is decreased. Also at negative E_h values, however, when no pre-oxidized quinone from the pool is present in the membrane, the rate does not decline significantly from its maximum value. (5) The size of the UQ pool affects also the number of charges translocated per flash by the bc_1 complex, since it affects the probability of rapid multiple turnover of the complex, which occurs at $E_h < 160$ mV in unextracted membranes. (6) These data are interpreted as evidence that oxidized UQ from the pool interacts collisionally with the Q_C site. At $E_h < 40$ mV, when the pool is totally reduced before the flash, the oxidized UQ molecule produced at every turnover at the Q_Z site

* Permanent address: Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina.

Abbreviations: BChl, bacteriochlorophyll; Mops, 4-morpholinepropanesulfonic acid; RC, reaction center; UQ, ubiquinone; UQH_2, ubiquinol; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine.

Correspondence: G. Venturoli, Dipartimento di Biologia, Istituto ed Orto Botanico, Università di Bologna, via Irnerio 42, 40126 Bologna, Italy.

of the bc_1 complex is sufficient for sustaining a rather high reaction rate at the Q_C site. The apparent K_m estimated for quinone reduction at the Q_C site is approx. 1 mM, less than one order of magnitude smaller than that estimated for QH_2 at the Q_Z site. The rate of transfer of ubiquinone from Q_Z to Q_C required by such a Q cycle mechanism does not appear incompatible with the experimental data.

Introduction

Chromatophores from non-sulphur purple bacteria contain a ubiquinol-cytochrome c_2 oxidoreductase complex (bc_1 complex) analogous in structure and function to that operating in mitochondria, chloroplasts and some aerobic bacteria. This complex contains, on a single apoprotein, two *b*-type hemes (*b*-561, $E_{m,7} = 50$ mV in *Rhodobacter sphaeroides* [1] and in *Rhodobacter capsulatus* [2]; *b*-566, $E_{m,7} = -90$ mV and -120 mV to -140 mV in *Rb. sphaeroides* [1] and *Rb. capsulatus* [2], respectively); it contains also one *c*-type cytochrome (cytochrome c_1, $E_{m,7} = 265$ mV and 285 mV in *Rb. Sphaeroides* and *Rb. capsulatus,* respectively [1,2]) and an Fe_2S_2 protein ($E_{m,7} = 285$ mV and 310 mV in *Rb. sphaeroides* [3] and *Rb. capsulatus* [3,4], respectively) (for a comparative review, see Ref. 5). In bacterial chromatophores, reducing and oxidizing substrates (ubiquinol-10 and ferricytochrome c_2, respectively) can be supplied to the bc_1 complex following photoactivation of the photosynthetic reaction center complex. A cyclic electron-transfer pathway is thereby established, whose kinetics have been accurately resolved by rapid flash spectrophotometry performed under conditions of strictly controlled ambient redox potential (for reviews, see Ref. 6–8). The usefulness of this system for investigating the function of the bc_1 complex is further increased by the possibility of studying the reaction steps involved in separation of electrical charge across the membrane. This can be accomplished by monitoring the associated electrochromic effects on the endogenous carotenoids produced by the electrogenic reactions (see, e.g., Refs. 9–13) and additional work reviewed in Ref. 14).

A modified Q-cycle model has been proposed on the basis of detailed kinetic studies of this system [15]. This model assumes the presence of three physically separated catalytic sites on the bc_1 complex: one (Q_Z or Q_O) site at which ubiquinol is oxidized by the Fe_2S_2 protein and by cytochrome *b*-566, in a concerted reaction; a second (Q_C or Q_I) site at which quinone is reduced by cytochrome *b*-561; a third site at which soluble ferricytochrome c_2 is reduced by cytochrome c_1. Two electron transfer steps within the complex couple these three reaction sites: the reduction of cytochrome *b*-561 by cytochrome *b*-566 and the reduction of cytochrome c_1 by the Fe_2S_2 center. On the basis of the electrochromic effects and in agreement with structural data, only electron transfer through the *b*-cytochrome chain appears to promote a transmembrane charge separation; electron transfer through Fe_2S_2 and cytochrome c_1 is catalyzed by proteins placed on the same side of the membrane as cytochrome c_2 [16]. The electrogenic steps occur on reduction of cytochrome *b*-561 by cytochrome *b*-566 and on electron transfer from ferrocytochrome *b*-561 to quinone or semiquinone at the Q_C site [16]. These electrogenic reactions have been identified on the basis of the sensitivity of the electrochromic signal to two specific inhibitors of the bc_1 complex, operating at two different sites: myxothiazol, which blocks quinol oxidation at the Q_Z site and inhibits completely the electrochromic signal associated with the function of the bc_1 complex; antimycin, blocking electron transfer from ferrocytochrome *b*-561 to quinone or semiquinone at the Q_C site, which, however, inhibits only partially the generation of an electric field across the membrane.

The reaction of ubiquinol at the Q_Z site has been analyzed in detail by studying the kinetics of cytochrome *b*-561 reduction induced by a single turnover flash in the presence of antimycin [15]. The reaction has been shown to follow a second order collisional mechanism [15], saturable with a K_m of 6–8 mM quinol in the membrane lipid phase [17,18]. The validity of this model has been strongly supported by measurements of the rate of this reaction over a very large range of quinol concentration; this has been made possible by

extracting or incorporating known amounts of ubiquinone in the membrane as well as controlling the ambient redox potential [18].

Evidence for the existence of a Q_C site, functionally and structurally different from the Q_Z site, has been presented in *Rb. sphaeroides*. Following previous analogous work in mitochondria [19,20] an antimycin sensitive radical signal has been detected by EPR in chromatophores at pH > 8.0 [21]. This signal has been attributed to Q_C and from its titration as a function of E_h and pH it has been concluded that UQH_2 binds about tenfold more strongly at the Q_C site than UQ and that at the Q_C site the semiquinone anion is strongly stabilized. Moreover, support for the existence of different catalytic sites interacting with UQH_2 in the bc_1 complex is based on the demonstration of two different routes for reduction of cytochrome *b*-561 [22,23], respectively inhibited specifically by myxothiazol or antimycin and by the isolation of the mutant strain R126 of *Rb. capsulatus* specifically blocked only at the Q_Z site [24].

The kinetics at the Q_C site have been much less studied. In fact the antimycin sensitive reversed electron transfer from quinol ($E_{m,7} = 90$ mV) at the Q_C site, to cytochrome *b*-561 ($E_{m,7} = 50$ mV), in the presence of myxothiazol, is not favoured thermodynamically when the quinone pool is largely oxidized; on the other hand, the redox buffer effect due to the large quinone pool prevents significant flash-induced redox changes in the system, when the quinone pool is partially pre-reduced. Nevertheless, reversed electron transfer at the Q_C site has been observed at alkaline pH (approx. 10) where the equilibrium constant is not so unfavourable, or at neutral pH in quinone extracted membranes, where the redox buffer effect was removed [22,23].

Measurements of the forward electron transfer at the Q_C-site in the absence of inhibitors is not simple, since reduced cytochrome *b*-561 is not accumulated to a significant extent after a flash [25]; this demonstrates that within the complex the rate of electron transfer to and from cytochrome *b*-561 are approximately equal. In principle, however, the kinetics of electrogenic steps from cytochrome *b*-566 to the Q_C site can be assayed in inhibited chromatophores by following the associated electrochromic effects [16].

In this study the involvement of quinone on the kinetics of the electrogenic reactions within the bc_1 complex has been investigated. It has been confirmed that, when the quinol concentration at the Q_Z site is limiting, the kinetics of the charge separation in the absence of inhibitors and of cytochrome *b*-561 reduction in the presence of antimycin A coincide. This study has been further extended to the kinetics of the electrogenic reactions at the Q_C site, under conditions in which the concentration of quinone is expected to become limiting. By analogy with our previous studies of quinol oxidation at the Q_Z site [18,26], this analysis has been extended to quinone extracted membranes.

Materials and Methods

A green strain of *Rhodobacter capsulatus* was grown photoheterotrophically with the medium described in [27], at a light intensity of approx. 40 $W \cdot m^{-2}$. Occasionally, in order to obtain chromatophore preparations characterized by a higher UQ/RC ratio [2], cells were grown at a higher light intensity (approx. 300 $W \cdot m^{-2}$) and harvested before the stationary phase, 10 h after inoculation. The bacterium utilized in this study was previously considered as a *Rhodobacter sphaeroides* strain [18,28,29]. The reattribution of this strain to a *Rhodobacter capsulatus* has been demonstrated by sequencing the fbc [30] (or pet [31,32]) operon by Davidson and Daldal and confirmed for the cultures used in our laboratory by tests of vitamin requirements during growth [33]. Chromatophores were prepared by French press disruption and differential centrifugation, as described in [34]. BChl was evaluated according to Clayton [35].

Kinetic spectrophotometric measurements were performed in a N_2 atmosphere under controlled redox conditions, as described previously [18]. All measurements were performed in 100 mM KCl, 50 mM Mops (pH 7.0) containing 2 μM nigericin and the following redox mediators: 1 μM each of phenazine methosulfate, phenazine ethosulfate and pyocyanin; 10 μM each of 1,2-naphthoquinone, 1,4-naphthoquinone, *p*-benzoquinone and duroquinone; and 2 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD). The concentration of chromatophores was adjusted so that a single flash

induced turnover in more than 90% of the reaction centers. Actinic flashes of light were provided by a xenon lamp (EG&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. The total concentration of photooxidizable reaction centers was measured from the change of absorbance at 542 nm, induced by a train of eight flashes, 20 ms apart, at $E_h = 160$ mV, in the presence of antimycin, using an extinction coefficient of 10.3 $mM^{-1} \cdot cm^{-1}$ [36,37]. Total photooxidizable cytochrome ($c_1 + c_2$) was measured at 551–542 nm following an analogous procedure and using an extinction coefficient equal to 19.5 $mM^{-1} \cdot cm^{-1}$ [36–38]. Cytochrome *b*-561 redox changes were measured at 561–569 nm, using an extinction coefficient of 19.5 $mM^{-1} \cdot cm^{-1}$ [15,37]. Measurements performed in the presence of antimycin A and myxothiazol (not shown) have demonstrated that any photoinduced signal due to RC and cytochrome ($c_1 + c_2$) redox changes is eliminated at the wavelength pair 561–569, also in chromatophore preparations from cells grown under the higher light intensity. Carotenoid electrochromic absorbance changes were monitored at 503 nm [16,39].

Lyophilization of chromatophores and extraction of ubiquinone were performed as in Ref. 18. The total membrane UQ content of the different lyophilized and partially UQ-extracted preparations was determined by exhaustive extraction and reversed phase HPLC analysis, as described in Ref. 18. Carotenoid content was estimated spectrophotometrically at 438 nm [18]. In UQ extracted chromatophores, the presence of UQ able to act as secondary acceptor (Q_B) in the reaction center complex, was assayed by analyzing the kinetics of charge recombination after a single flash, in the presence of antimycin, at $E_h = 430$ mV [26,40].

Results

Single turnover electrochromic response in quinone-extracted chromatophores

When dark-adapted chromatophores are activated by a single turnover flash, a carotenoid electrochromic signal can be detected formed by three different kinetic phases. The first two phases (I and II), that are completed within a few microseconds (and are not kinetically resolved in the traces shown in Fig. 1) are associated with electrogenic reactions taking place in the reaction center complex. On the basis of the topological information available on the RC complex [6,9–14,39,41,42], the overall amplitude of phase I plus phase II is considered to correspond to the electrical field produced by the translocation of one electron per RC photoactivated across the whole thickness of the membrane dielectric. In agreement with this idea, phase (I + II) is totally unaffected by electron-transfer inhibitors of the bc_1 complex (Fig. 1A) and titrates out in correspondence with the dark pre-reduction of Q_A [43].

A third slower phase (phase III) is also present, whose maximal extent at $E_h = 120$ mV is nearly as large as phase (I + II) (Fig. 1A). This electrochromic signal is thought to be related to the function of the bc_1 complex on the basis of the dependence of its rate and extent on the ambient redox potential and of its sensitivity to specific inhibitors [6,9–14,39,41]. This phase has been further resolved into two components, one sensitive and one insensitive to antimycin [16], as described in Introduction. Phase III is totally eliminated by myxothiazol.

In Fig. 1B, the electrochromic signal recorded in quinone-extracted membranes (containing a residual amount of quinone equal to 6 UQ per RC) is shown. The amplitude of phase (I + II) is slightly diminished. This effect is interpreted as due to the partial extraction of the carotenoids which, as reported previously [12,18,44], partition weakly into the isooctane phase on extraction of lyophilized chromatophores. This has been confirmed by a quantitative analysis of the membrane pigments, showing that the loss of carotenoids is proportional to the decrease in extent of phase (I + II) [18]. In contrast, the extraction of quinones affects both the rate and the extent of phase III, in agreement with the concept that this phase is related to electrogenic steps dependent on oxidation of the UQ pool by the bc_1 complex.

The amplitude of phase III gives information on the number of electrons transferred by the bc_1 complexes across the membrane; as discussed pre-

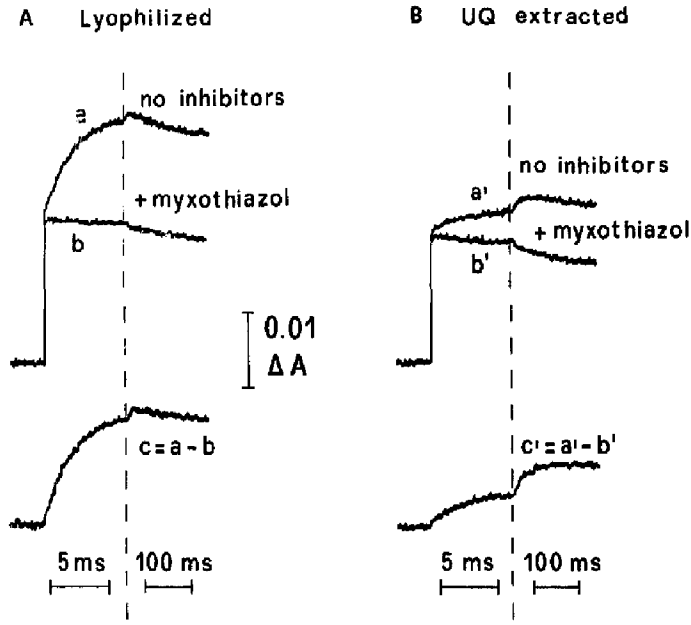

Fig. 1. Kinetics of the carotenoid electrochromic change induced by a single turnover flash. (A) Control-lyophilized chromatophores, characterized by 16 UQ/RC. (B) Partially UQ-extracted chromatophores (6 residual UQ/RC). The assay conditions are described under Materials and Methods. The concentration of BChl in the cuvette was 60 μM for both preparations; 3 μM myxothiazol was present where indicated. The ambient redox potential (E_h) was 120 mV. Each signal was recorded at two different sampling rates: the dashed lines indicate the sweep change-over point. Signals (average of two measurements with a time constant of 20 μs) show the absorbance change at 503 nm. Traces c and c′ are the differences between traces without inhibitor (a, a′) and traces in the presence of myxothiazol (b, b′) in control and UQ-extracted chromatophores, respectively. This myxothiazol-sensitive phase is referred to in the text as phase III of the carotenoid change.

viously [15,16], the number of charges translocated is related to the RC/bc_1 stoichiometric ratio in the membrane (approx. 2 under usual conditions of growth [2]) and to the number of turnovers of the bc_1 complex induced per flash. In line with these concepts, this amplitude is dependent on the redox potential: it is approximately only one-half of that of phase (I + II) at $E_h > 180$ mV, when, because the quinone pool is completely oxidized before the flash, the bc_1 complex can turn over rapidly only once; it becomes 75% of phase (I + II) at lower E_h values, when the availability of pre-reduced quinone in the pool allows a second turnover of the bc_1 complex per flash [16]. At very low E_h values, however, when part of the RC is inactivated by pre-reduction of Q_A before the flash, the turnover becomes limited by the decreased number of oxidizing equivalents generated per flash. This limitation of oxidizing equivalents occurs well before the limit of one oxidizing equivalent per bc_1 complex, since for thermodynamic reasons the Fe_2S_2 protein remains largely reduced when only one electron is removed from the high-potential chain of the complex [15]. Therefore, in order to resolve the dependence of the electrogenic events upon the redox state of the quinone pool over this E_h range, the electrochromic signals induced by a single flash have been normalized to the fraction of RC actually in operation.

The data presented in Fig. 2 show the titration of the extent of phase (I + II) in lyophilized-control chromatophores and in two quinone-extracted preparations measured in the presence of myxothiazol. At $E_h > 100$ mV the amplitude is E_h independent and is decreased only because of the partial extraction of carotenoids (cf. Ref. 18). The

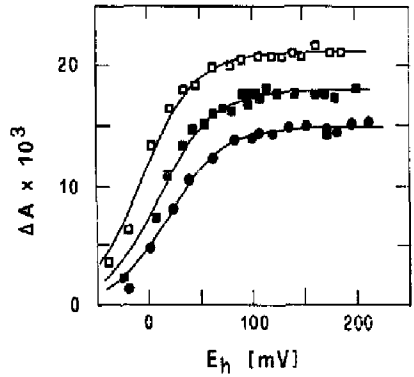

Fig. 2. Redox titrations of extent of the fast phase (phase I + II, myxothiazol-insensitive) of carotenoid change. The values of the absorption changes were taken from traces similar to those shown in Fig. 1. Experimental conditions were as described in Fig. 1. □——□, Control, lyophilized chromatophores (16 UQ/RC; 0.73 total photooxidizable cytochrome $(c_1 + c_2)$/RC); ■——■, UQ-extracted chromatophores (6 UQ/RC, 0.71 cytochrome $(c_1 + c_2)$/RC); ●——●, UQ-extracted chromatophores (4 UQ/RC, 0.69 cytochrome $(c_1 + c_2)$/RC). Kinetic analysis of the post-flash relaxation of oxidized BChl dimer at $E_h = 430$ mV (back reaction) revealed that 20% and 30% of Q_B had been removed in the UQ-extracted preparations characterized by 6 and 4 residual UQ/RC, respectively. For each titration, the best fitting Nernstian curve ($n = 1$) is drawn through the experimental points.

amplitude titrates out between 100 and −50 mV with a Nernstian behaviour: the apparent E_m values are −6 mV for control and 10 and 20 mV for the two extracted preparations, containing six and four residual quinones per RC, respectively. Measurements of the amplitude of the back reaction from Q_A^- to $[BChl]_2^+$ ($t_{1/2} \approx 50$ ms) in the two extracted preparations, indicated that Q_B was absent in 20 and 30% of centers respectively. The shift of the apparent E_m towards more positive values is presumably related to the removal of Q_B. In fact, in isolated reaction centers, removal of Q_B has been shown to induce a positive shift in the E_m value of the primary quinone acceptor [45]. Similar E_m shifts have been described previously in chromatophores from several bacterial species, as induced by ortophenantroline, an inhibitor of electron transfer from Q_A to Q_B [43,46]. The analogy between these two phenomena is presently under study.

The amplitude of phase (I + II) at the different values of E_h has been considered as a measure of the active RC, and utilized in the following analysis for normalizing the amplitudes and the rates of phase III in the correspondent preparations. This approach does not correspond to any gross approximation, since about 80% of the RC appears to be rapidly re-reduced after a flash in these lyophilized and UQ-extracted preparations and is therefore redox coupled with the bc_1 complex via cytochrome c_2 (not shown). It should be noted, however, that the presence of any amount of slowly reducible RC does not interfere with the traces of phase III, since this is obtained by subtraction of traces obtained in myxothiazol inhibited chromatophores. In the subtracted trace therefore any electrogenic signal due to myxothiazol insensitive charge-transfer phenomena and therefore bc_1 independent is included. For the present purpose the amplitude of phase (I + II) is only utilized in order to calibrate the amplitudes and rates of the electrochromic traces in terms of elementary charges on the basis of the notion that each RC photooxidized and re-reduced by cytochrome c_2 promotes the translocation of one electron across the entire membrane. The only error in this calibration could derive by the existence in the membrane preparation of photooxidizable RC's electrochromically silent because located in uncoupled non-vesicular systems or in inversely oriented and therefore contributing negatively to the electrochromic traces. This latter case is, however, quite unlikely, since these misoriented RC's cannot be reduced rapidly by internal cytochrome c_2, in contrast to our experimental evidence. These conclusions are even more stringent for the chromatophore preparation utilized for the experiment of Fig. 5 (see below).

Fig. 3 presents redox titrations of the amplitude of phase III of the carotenoid band-shift, induced by one single turnover flash, recorded in lyophilized-control and UQ-extracted chromatophores. The experimental points present were obtained by subtracting from traces recorded in the absence of inhibitors (such as traces a, a′ in Fig. 1) traces obtained in the presence of myxothiazol (such as traces b, b′ in Fig. 1) at the same value of E_h (±5 mV) recorded in a subsequent titration of the same sample. As discussed above, the amplitudes

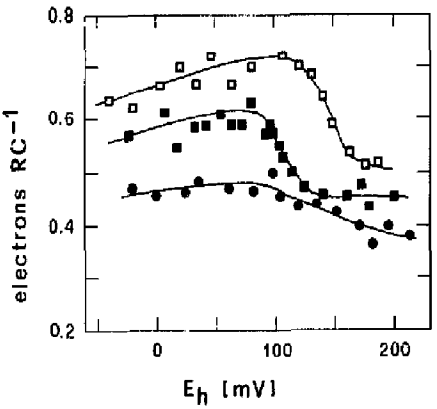

Fig. 3. Redox titrations of the extent of phase III of the carotenoid bandshift in control-lyophilized and UQ-extracted chromatophores. Symbols as in Fig. 2. The extents were normalized to the fraction of RC turning over and were expressed as electrons per RC on the basis of the data shown in Fig. 2 (see text for details).

of the experimental traces have been corrected for the fraction of carotenoid extracted and, at low E_h values, for the fraction of RC inactivated by the pre-reduction of Q_A. The amplitudes are, therefore, expressed as electrons translocated per photo-activated RC complex. In control chromatophores the amplitude is a function of E_h, as previously described [15,16], and becomes about 1.5 times larger in extent when E_h is lowered from approx. 200 to 100 mV. The increase in amplitude over this redox span has been interpreted as a progressive induction of a second rapid turnover of the bc_1 complex, when more than one quinol per bc_1 becomes available in the membrane after the flash. A similar increase in amplitude is also observed in extracted chromatophores. The stimulation in the extent titrates in, however, at much lower E_h values (around 100 mV) and the maximal amplitude is markedly diminished. In the more extensively extracted chromatophores (4 UQ/RC), the extent becomes nearly E_h independent, and reflects a turnover of less than 0.5 electrons per RC. This behaviour is consistent with the nearly complete extraction of the UQ pool (since a large proportion of the residual quinones is proposed to be bound to protein complexes (cf. Refs. 18 and 47)) and with the partial extraction of Q_B. These data are in agreement with the result of direct measurements of the amount of cytochrome *b*-561 photoreduced in drastically UQ extracted chromatophores [18]. In all cases, the normalized extents do not decrease significantly at $E_h < 90$ mV, where the turnover of the bc_1 complex could be expected to become progressively limited by the concentration of oxidized quinone at the Q_C site.

The redox titrations of the initial rate of phase III of the carotenoid bandshift (measuring the rate of charge separation taking place while the bc_1 complex undergoes the first turnover) are presented in Fig. 4 for control and extracted chromatophores. The rates plotted correspond to the initial fast rates measured neglecting the short lag, which has been attributed to redox reactions occurring at the Q_Z site and within the FeS-cytochrome c_1 portion of the bc_1 complex. As with the extents, the values of the rate, that are expressed as electrons per photo-activated RC per ms, have been corrected for the fraction of reaction centers inactivated owing to the pre-reduction of Q_A. As previously described [16], in control chromato-

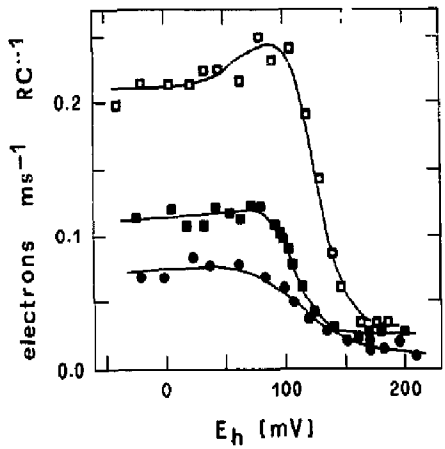

Fig. 4. Initial rates of phase III of the carotenoid absorbance change as a function of redox potential in control-lyophilized and UQ-extracted chromatophores. Symbols as in Fig. 2. The rates were corrected as in Fig. 3 for the fraction of RC inactivated by the pre-reduction of Q_A by a calibration against the extent of phase (I+II), measured in the same experiments (Fig. 2) (see text for details). The rates are expressed in units normalized to the total amount of photooxidizable RC measured in the same sample.

phores, the rate of charge separation is very steeply increased when the E_h is lowered between 200 and 100 mV. At low E_h, between 100 and −50 mV, the corrected rate is always very high, diminishing by no more than 20% of the maximum experimental value. In extracted chromatophores, characterized by six residual UQ per RC, the rate at high E_h is almost equal to that in the control and is increased upon reduction of the residual UQ pool. The stimulation of the rate is, however, markedly lower than in control, and, most importantly, begins at a much lower E_h value. These effects are further enhanced in more drastically extracted chromatophores (4 UQ/RC). In this preparation the rate at high E_h values is inhibited by 50%, in accordance with the partial extraction of Q_B and with what has been previously described for the photoreduction of cytochrome *b*-561 in the presence of antimycin and uncouplers [18]. Also in extracted chromatophores the maximal rate of charge separation, which is reached around 70 mV, is not decreased at lower E_h values, when the small residual pool is expected to be practically all reduced before the flash. Reconstitution of the extracted pool with exogenous UQ-10 [18] largely reverts the effects of UQ extraction both on the extent and on the rate of phase III, when normalized to the fraction of unextracted carotenoids (not shown, compare also Ref. 44). Although a complete kinetic analysis has not been performed, it is expected that the reconstitution of a fast phase III will parallel that of a fast reduction of cytochrome *b*-561 as studied in details in Ref. 18.

Direct comparison of the reaction rate at the quinol oxidizing site and of the initial rate of charge separation

The trend of the initial rate of charge separation as a function of E_h presented in Fig. 4 is related to the nature of the kinetic limiting step in the overall electrogenic reaction within the bc_1 complex under different redox poise. Information on this point can be gained from a direct comparison, in the same preparation, of the initial rate of reduction of cytochrome *b*-561 induced by a single flash, measured in the presence of antimycin and uncouplers, and of the initial rate of phase III of the electrochromic signal measured in the absence of uncouplers. The addition of uncouplers for the measurement of cytochrome *b*-561 redox kinetics is needed for preventing interference by electrochromic signals [48]. No large kinetic effect due to the addition of uncouplers is expected, since the first turnover of the bc_1 complex should occur in the presence of a small membrane potential. This assumption is supported by the negligible effects of the membrane potential on the rate of RC re-reduction and of cytochrome *b*-566 reduction after the first flash, demonstrated by comparing kinetic traces in coupled and uncoupled chromatophores after correcting for electrochromic interference (cf. Ref. 48, Figs. 4 and 5). The initial rate of reduction of cytochrome *b*-561, measured in uncoupled conditions, has been shown to be controlled by the rate of quinol oxidation at the Q_Z site, until a maximum, when the measured rate becomes limited by the pre-reduction of cytochrome *b*-561 before the flash [15,17,18]. This rate can be generally described by a Michaelis-Menten behaviour vs. UQH_2 concentration, when corrected for cytochrome *b*-561 pre-reduction [18]. A redox titration of the initial rate of cytochrome *b*-561 is presented in Fig. 5 (lower panel). As described in Refs. 15, 17 and 18, under these conditions, the maximal rate of cytochrome *b*-561 reduction is measured after a lag (from 0.1 to 0.8 ms, depending on the E_h); this lag and its dependence on E_h has been interpreted to be related to the time required for the transfer of UQH_2 to the Q_Z site and to the photooxidation of the FeS-cytochrome c_1 chain. For the purpose of the analysis of Fig. 5, the maximal rate after the lag has been considered, following the procedure already described in Refs. 17 and 18. For these measurements a chromatophore preparation from cells grown at high light intensity has been utilized (see Materials and Methods). In this type of cells a higher synthesis of cytochrome c_2, of bc_1 complex and of ubiquinone with respect to that of RC is induced as a result of adaptation to high light intensity [2]. Chromatophores from such cells contain consequently a large amount of cytochrome c_2 and of bc_1 complex per RC (compare the legend of Fig. 5 and traces c, d, e of Fig. 6). A fast, complete re-reduction of RC and of cytochrome $(c_1 + c_2)$ after each flash in a train is therefore observed (Fig. 6a, b). Moreover, in this

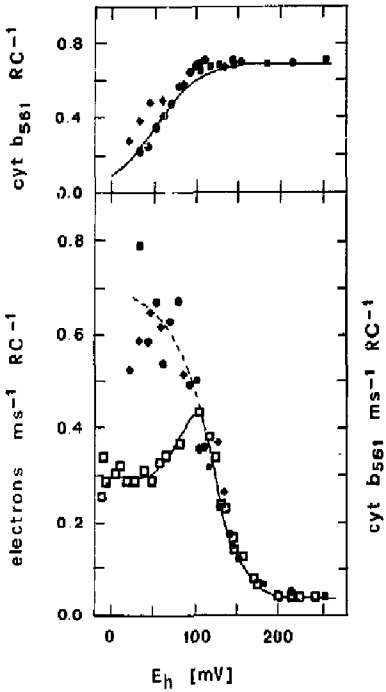

Fig. 5. Direct comparison between the initial rates of phase III of the carotenoid signal (□-----□) and of the reduction of cytochrome (cyt) *b*-561 (●-----●, ■-----■, ◆-----◆), measured in the same membrane preparation, as a function of the redox potential (lower panel). The different closed symbols in the titration curve of cytochrome *b*-561 reduction rate refer to independent sets of measurements performed on the same membrane preparation. Antimycin and valinomycin (10 μM each) were added to the sample for the measurements of cytochrome *b*-561 reduction rate. The upper panel show the maximum extent of cytochrome *b*-561 reduction induced by a single flash and evaluated from the same kinetic traces. Also in this case, as for Fig. 4, the data were normalized to the total amount of photooxidizable RC. These data were used for a normalization of the rate of reduction of cytochrome *b*-561 at $E_h < 90$ mV (see text). As in Fig. 4 the rates of the carotenoid signal were corrected for the fraction of Q_A pre-reduced before the flash. The chromatophore preparation utilized for these measurements was purified from cells grown at high light intensities and was characterized by a total UQ complement of 30 UQ/RC. The total amount of photoxidizable cytochrome ($c_1 + c_2$) and of photoreducible cytochrome *b*-561 were 1.4 and 0.85 per RC, respectively.

preparation the natural abundance of the quinone pool [2,29] produces at $E_h < 170$ mV a fast turnover at the Q_Z site. Below $E_h = 90$ mV, the experimental data have been normalized to the maximum extent of cytochrome *b*-561 reduced per RC following a single flash. The redox titration of the extent (Fig. 5, upper panel) has been obtained from the same kinetic traces utilized for the measurement of the initial rate. This type of correction

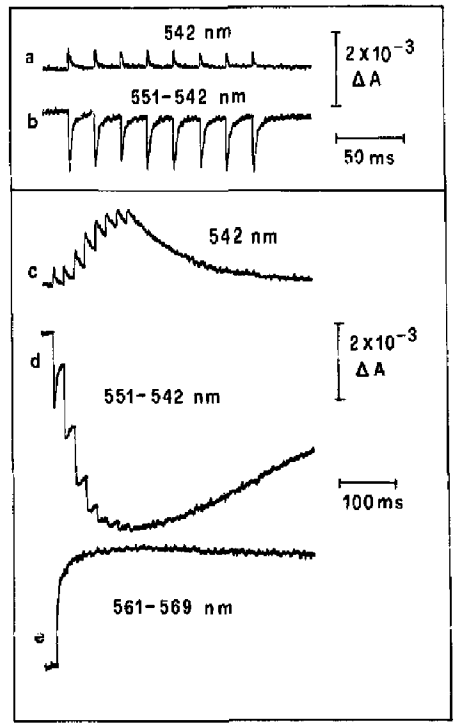

Fig. 6. Changes in the redox state of reaction center (traces a and c), cytochrome ($c_1 + c_2$) (traces b and d) and cytochrome *b*-561 (trace e) induced by a train of eight flashes, fired 20 ms apart. Chromatophores from the same membrane preparation utilized for the measurements of Fig. 5 were suspended in 50 mM Mops buffer (pH 7.0) containing 100 mM KCl to a reaction center concentration of 0.32 μM, in the presence of 2 μM nigericin and valinomycin. Traces a and b: chromatophores were suspended in an open, unstirred cuvette, in the presence of 1 mM sodium ascorbate and 1 mM potassium cyanide. Traces (200 ms sweep, 200 μs RC) are the average of eight measurements with a dark time of 1 min between trains of flashes. Traces c, d and e: measurements were performed in a potentiometric cuvette in a N_2 atmosphere, in the presence of the redox mediators detailed under Materials and Methods and of 10 μM antimycin. The ambient redox potential was poised at $E_h = 160$ mV. Traces are the average of four measurements, taken with a sweep of 500 ms and an instrument response time of 500 μs.

takes into account simultaneously the pre-reduction of cytochrome *b*-561 and, at very low E_h values, also the partial pre-reduction of Q_A in the reaction center. After this correction, therefore, the kinetic data reflect only the turnover of the Q_Z site per active bc_1 complex. A redox titration of the initial rate of phase III, obtained in the same preparation, in the absence of uncoupler, is also presented in Fig. 5 (lower panel), normalized as in Fig. 4 to the fraction of active RC's. The ratio (membrane current/cytochrome *b*-561 reduction rate) is exactly one electron per cytochrome *b*-561 reduced between $E_h = 260$ mV and $E_h = 100$ mV. Below this value the rate of charge separation declines and becomes constant at E_h lower than approx. 40 mV. It is evident therefore that the initial rate of charge separation across the whole membrane can become significantly lower than that of the electron delivery to cytochrome *b*-561 when the reduction of a large UQ pool renders large concentrations of UQH_2 available at the Q_Z site and progressively decreases the availability of oxidized quinone at the Q_C site.

Discussion

The kinetic model underlying the interpretation of the present data is the modified Q-cycle previously presented [15], and the notion that phase III of the carotenoid bandshift is associated with two electrogenic steps within the bc_1 complex, including the reduction of quinone at the Q_C site [16]. According to the model, the interaction of quinone with the bc_1 complex occurs at two kinetically distinguishable sites. At the quinol oxidizing site (Q_Z), the rate of reaction has been demonstrated to obey a second order Michaelis-Menten type mechanism [15,17,18]. At high E_h, when the concentration of quinol in the membrane is much lower than the K_m, the rate of the reaction can be written as a simple second-order rate equation of the type

$$v_z = k_z[QH_2][FeS^+ \cdot \text{cytochrome } b\text{-}566 \cdot \text{cytochrome } b\text{-}561] \quad (1)$$

At low E_h values, the quinol concentration is not limiting; however, in particular below 70 mV when a portion of the reaction centers becomes inactivated, due to the pre-reduction of Q_A, the measured rate at Q_Z and number of turnovers of the bc_1 complex induced per flash, are strictly controlled by the fraction of reaction center turning over, i.e., by the number of electron-accepting carriers produced per flash. This phenomenon has been taken into account by the normalization against the extent of phase (I + II).

By analogy, the reduction of quinone at the Q_C site could be expected to take the form

$$v_c = k_c[Q][Q_CH_2 \cdot (\text{cytochrome } b\text{-}566^- \cdot \text{cytochrome } b\text{-}561^-)] \quad (2)$$

where Q is oxidized, quinone from the pool (including Q released by the Q_Z site) and $[Q_CH_2 \cdot (\text{cytochrome } b\text{-}566^- \cdot \text{cytochrome } b\text{-}561^-)]$ indicates the totally reduced cytochrome *b* chain in which the Q_C site is occupied by reduced quinone. This means that a quinol molecule has been assumed to be bound at the Q_C site before the flash and to exchange with an oxidized quinone molecule present in the pool in order to allow the oxidation of *b*-cytochromes: this is the most likely situation when, at low E_h, the large UQ pool is prevalently reduced and is consistent with the thermodynamic properties of the Q/Q^- and Q^-/QH_2 coupled bound to the Q_C site, as evaluated from the behaviour of the Q_C EPR signal [21]. Whatever the real status of the Q_C site may be, Eqn. 2 is intended merely to indicate the bimolecular nature of the reaction at the Q_C site and does not account for the saturation phenomena dependent on the concentration of oxidized quinone from the pool ([Q]) (see below). Other second-order reaction schemes involving oxidized quinone from the pool can be considered at more positive E_h values [7]. At any equilibrium E_h in the dark, the extra electron which enters the cytochrome *b* chain following the flash and becomes available at the Q_C site, is strictly related to the turnover of the Q_Z site, and, therefore, again related to the fraction of reaction centers turning over and redox coupled to the bc_1 complex [1,7,15]. For this reason, in order to resolve the dependence of the reaction rate at the Q_C site on the concentration of quinone [Q], we have normalized the

experimental rates and extents of phase III of the carotenoid bandshift to the fraction of active RC, when Q_A is partially pre-reduced. No other correction parameter was introduced, either in control or in UQ extracted membranes, since the extraction procedure did not decrease significantly the content of cytochrome ($c_1 + c_2$) and since the rate of the back reaction in Q_B-extracted reaction centers ($t_{1/2} \simeq 50$ ms [26]) does not compete efficiently with the re-reduction of the reaction center by cytochrome ($c_1 + c_2$) [1].

The turnover at the Q_Z site

In antimycin inhibited and uncoupled chromatophores, poised at high redox potential ($E_h >$ 150 mV at pH 7), the rate of flash-induced reduction of cytochrome *b*-561 is strictly controlled by the turnover of the Q_Z site, since the concentration of quinol (either pre-reduced before the flash, or generated by the RC) is kinetically limiting [15]. In uninhibited chromatophores, the electron transfer through the cytochrome *b* chain of the complex appears to be very fast [25] and can be directly monitored by the electrochromic signal [16]. Also in the absence of inhibitors, at high E_h values, the rate is expected to be controlled by the turnover of the Q_Z site, as discussed thoroughly in Ref. 16. The titration of the initial rate of charge transfer (Fig. 4) fully supports this expectation: the rate, which in control chromatophores is increased at $E_h < 160$ mV, is stimulated in a similar fashion in UQ extracted chromatophores (6 UQ/RC) only at $E_h < 130$ mV. This behaviour is enhanced in chromatophores more drastically extracted (4 UQ/RC). The absolute rates of phase III in unextracted and extracted preparations become comparable when expressed as a function of the absolute quinol concentration in the membrane (not shown), if it is assumed that part of the native UQ content (4–5 UQ/RC [47]) is not freely diffusable in the membrane and is not extracted by the isooctane treatment (cf. Ref. 18). This phenomenon coincides with that previously demonstrated in UQ-extracted, uncoupled chromatophores for the reduction of cytochrome *b*-561 in the presence of antimycin [18]. When the absolute rates of phase III charge transfer, measured at $E_h > 110$ mV and calibrated against the extent of phase (I + II), are compared with the rate of cytochrome *b*-561 reduction in the presence of antimycin and uncouplers, in chromatophores with a comparable size of the UQ pool and at the same E_h (cf. Ref. 18), a fairly constant value around 1 can be calculated for the ratio (membrane current/cytochrome *b*-561 reduction rate). In the experiments described in Fig. 5 the rate of charge transfer and of cytochrome *b*-561 reduction are directly compared in the same membrane preparation; the data support the absolute coincidence of the two processes. The overlapping of the results of these two sets of measurements also supports the accuracy of the extinction coefficients utilized to evaluate the reduction of cytochrome *b*-561 and the reliability of the calibration procedure utilized for the estimation of the membrane current. The coincidence in rates only holds for $E_h > 100$ mV, below which it is interpreted that the kinetic interference of the reactions at the Q_C site starts to become relevant (see below). These results are therefore consistent with a model which assumes that for one QH_2 oxidized, one electron is transferred by the bc_1 complex across the entire membrane dielectric, with a rate controlled by the reaction step at the Q_Z site [16].

The consequences of the quinone extraction on the extent of phase III are also in support of the model. The stimulation of the extent, which is related to multiple turnover of the bc_1 complex after the flash and is therefore controlled by the availability of quinol in the pool, is shifted to more negative E_h values in UQ extracted as compared to control chromatophores. The maximum extent is also reduced when the size of the UQ pool is decreased, reflecting the diminished probability of multiple rapid turnovers when the UQ concentration in the membrane is lowered. However, in both control and extracted chromatophores, the maximum extent is consistently lower than the theoretical maximum of two turnovers per flash (since two electron holes per bc_1 complex should be produced per flash by the two reaction centers present per bc_1 in the membrane), in agreement with previous data [16]. The possible reasons of this discrepancy have been already discussed [16].

The turnover at the Q_C site

At pH 7.0 and at $E_h < 30$ mV the quinone is

approx. 99% reduced and cytochrome *b*-561 is 70% pre-reduced before the flash. Following the Q cycle reaction scheme, upon flash excitation, the photooxidized $[BChl]_2^+$ of the RC causes, through the action of cytochrome c_2, a rapid oxidation of the [FeS-cytochrome c_1] chain of the complex and injects one electron in the [*b*-566-*b*-561] chain. A rapid electron transfer from cytochrome c_1 to the photooxidized RC in the absence of cytochrome c_2 has been, however, suggested by Prince et al. [49] and Prince and Daldal [50]. This cytochrome *b* doublet, which is now predominantly reduced, acts as a reductant of quinone or semiquinone at the Q_C site. Since according to the Q cycle scheme the redox coupling between the oxidation of the FeS-cytochrome c_1 chain and the reduction of the *b* cytochromes is complete, the concentration of the reductant at Q_C is related to the turnover of the Q_Z site (and of the RC complexes). The dependence of the reaction rate in the second-order process of Eqn. 2 on [cytochrome *b*-566$^-$ · cytochrome *b*-561$^-$] has been taken into account by the normalization procedure against phase (I + II) used in the presentation of rates in Figs. 4 and 5; the dependence of the rate on the remaining variable in Eqn. 2 (i.e., the concentration of oxidized quinone) should become explicit in the plots.

When cytochrome *b*-561 is pre-reduced before the flash, the first electrogenic step should coincide with the oxidation of cytochrome *b*-561 at the Q_C site. The subsequent re-reduction of cytochrome *b*-561 completes the transfer of one electron across the membrane. The data of Fig. 5 demonstrate that the rate of the entire process is strongly limited by the turnover of the Q_C site, since the rate of charge translocation is much lower than the rate of cytochrome *b*-561 reduction measured in the presence of antimycin and valinomycin (and normalized to the total cytochrome *b*-561 in order to evaluate the turnover rate of the Q_Z site). Below $E_h = 110$ mV the normalized rate of cytochrome *b*-561 reduction and of charge separation progressively diverge. In the intermediate range of E_h (30 mV < E_h < 110 mV), since the kinetics of carotenoid phase III results from the convolution of the two electrogenic steps of cytochrome *b*-561 reduction and oxidation, clearly the rate at the Q_C site cannot exceed a maximum value coincident with the point of divergence with the rate of turnover at the Q_Z site. The value of the rate of charge transfer at the divergence point therefore allows to estimate the turnover rate at Q_C for that given E_h value and in relation to the size of the UQ pool in the membrane. In the case of the data of Fig. 5, the divergence point occurs at $E_h = 108$ mV, that correspond to 80% oxidized quinone in the pool or about 40 mM UQ oxidized in the membrane (in the preparation of Fig. 5 the actual size of the UQ pool was estimated in the order of 25 UQ/RC, corresponding to about 50 mM in the membrane [17].

The rate of charge transfer progressively declines at lower E_h values upon further reduction of the UQ pool and levels to a constant, E_h independent value, below $E_h = 40$ mV. In the Q-cycle mechanism adopted as a model, one molecule of oxidized quinone is generated at the Q_Z site per turnover of the bc_1 complex. This sets a lower limit to $[Q_{ox}]$, which is a function of E_h, i.e., of the number of active RC, over the range below $E_h = 40$ mV at which the pool becomes fully reduced. The availability of this particular molecule of oxidized quinone at the Q_C site requires its rapid transfer from the Q_Z site. This must take place with a rate at least as fast as that measured for the phase III kinetics at these E_h values ($t_{1/2} \approx 2$ ms). This conclusion is not invalidated by the fact that the whole of the phase III signal includes charge-transfer events taking place in two consecutive redox reactions, since no marked biphasic kinetics can be detected. At $E_h < 40$ mV the overall rate of phase III is therefore the minimum rate of charge transfer at the Q_C site accessible to experimentation. The rate of charge transfer at $E_h < 40$ mV decreases to 0.3 $e^- \cdot RC^{-1} \cdot ms^{-1}$ as compared to the value of 0.43 $e^- \cdot RC^{-1} \cdot ms^{-1}$ measured at the divergence point. At $E_h <$ 40 mV $[Q_{ox}]$ should be about 1 mM in the membrane phase, since the concentration of RC in the lipid is approx. 2 mM [17]. Assuming a Michaelis-Menten kinetics at the Q_C site the two sets of rates and $[Q_{ox}]$ at the divergence point and at $E_h < 40$ mV, would correspond to a K_m of approx. 0.5 molecules of oxidized quinone per RC (or about 1 mM oxidized quinone in the membrane phase). It should be noted, however, that

the treatment of electron transfer at the Q_C site in terms of Michaelis-Menten kinetics can be inaccurate, particularly when $[Q_{ox}]$ becomes very limiting, since this approach assumes implicitly an immediate availability of Q_{ox} at the site. However, when the only ubiquinone in the membrane is that produced at the Q_Z site, the slow kinetics at the Q_C site can conceivably be limited by the transfer of UQ from Q_Z to Q_C. Therefore the Michaelis-Menten description can be considered strictly valid only if the dissociation process of UQ from the Q_Z site and its diffusion to the Q_C site are never rate limiting. The estimated K_m value for quinone at the Q_C site is approx. 8-fold smaller than the K_m for quinol at the Q_Z site, that was evaluated around 8 mM from the redox titration of the rate of cytochrome *b*-561 reduction in the same preparation (cf. also Ref. 18). When the quinone pool is fully reduced before the flash the concentration of oxidized quinone available at Q_C should be identical to that of the bc_1 complexes photoactivated. Therefore the concentration is in the order of the K_m. The second order approximation for the reaction kinetics of Eqn. 2 is not fully justified, given this value of the K_m and considered that the minimal experimental concentration of oxidized quinone cannot be lower than the concentration of the bc_1 complexes activated by the reaction centers. In any case, considering for calculations the experimental initial rates, a minimum value fo the second order kinetic constant would be approximately $k_C = 3 \cdot 10^5\ M^{-1} \cdot s^{-1}$. With similar reasoning a second-order rate constant at the Q_Z site $k_Z = 6 \cdot 10^4\ M^{-1} \cdot s^{-1}$ can be evaluated from Fig. 5. This last value is in far, but not complete agreement with previously reported rate constants; the difference reflects a variability of the rates found for different preparations. Similar variability was detected for the reduction rate of cytochrome *b*-561 in the presence of antimycin [15,17,18] and is also evident when the experimental values in Fig. 4 and Fig. 5 are compared.

The behaviour of phase III kinetics in UQ extracted chromatophores (6 UQ/RC), Fig. 4, appears to support the general mechanism outlined above. The stimulation of the rate of charge transfer titrates in at significantly lower E_h values, reflecting the response of the reaction rate at the Q_Z site to the absolute quinol concentration in the lipid phase [15,17,18]. At E_h below 70–80 mV the rate reaches a maximum and remains constant down to −30 mV. The value of the rate is always much lower than that in unextracted chromatophores, indicating that, for the whole E_h span examined, the turnover at the Q_Z site always controls kinetically the rate of phase III. The constancy at the maximum value of the rate at low E_h values in extracted preparations differs significantly from the behaviour observed in unextracted membranes, for which a decrease below the maximum value is observed at low E_h values, where the only oxidant available is the quinone produced at the Q_Z site.

The physically separate identity of the Q_Z and Q_C sites in the complex is a specific feature of any Q-cycle scheme. As described in the Introduction the support to this idea comes from the evidence of different routes for cytochrome *b*-561 reduction [22,23] and from the observation that different inhibitors block specifically one of these route [51,52]. The existence of two sites is also consistent with the characterization of photosynthetic mutants impaired only at the Q_Z site and is compatible with recent structural models, based on the primary aminoacid sequence of the subunits of the bc_1 complex [53,54] and with the locations in one of these models [54] of several amino acid substitutions conferring inhibitor resistance. Based on redox and pH titrations of Q_C, as detected by EPR spectroscopy, the midpoint potential of the UQ/UQH_2 couple bound to the Q_C site was evaluated to be 150 mV at pH 7.0, some 60 mV more positive than the $E_{m,7}$ of the UQ pool. This indicates a preferential binding of QH_2 at the Q_C site, but does not exclude in principle a rapid exchange of both the reduced and oxidized species with the pool. The preferential binding of UQH_2 at Q_C reinforces our assumption that, particularly at low E_h values, the most likely mechanism for the oxidation of cytochrome *b*-561, must include an exchange of UQ_CH_2 with UQ from the pool (Eqn. 2). Moreover, under our experimental conditions (pH 7.0), the EPR signal attributed to Q_C is reduced to undetectable levels. Alternative models (*b* cycle and its updated version, the semiquinone cycle) place the location of the two sites in a single hydrophobic pocket, within which the semi-

quinone produced at the Q_Z site is displaced to the Q_C site and acts as oxidant for ferrocytochrome *b*-561 [55,56]. In this case, the oxidant is produced within the complex itself and the antimycin-sensitive electrogenic step is proposed to coincide with the dislocation of the semiquinone anion [56]. Although the present experiments were not specifically addressed to discriminating between these two types of mechanisms, they strongly support the notion that the Q_C site interacts with a collisional mechanism with oxidized quinone from the pool, with a kinetic behaviour which appears to be independent of that at the Q_Z site. This conclusion appears to us only compatible with a Q-cycle type reaction scheme, since it indicates that the Q_Z and Q_C sites, although coupled through the *b*-cytochrome chain, are not only structurally but also kinetically independent. In fact the data demonstrate that when the turnover at the Q_C site becomes limiting in the overall turnover of the bc_1 complex, the rate of electron transfer through the complex becomes sensitive to the degree of oxidation of the UQ pool. Our observations agree also with those drawn by De Vries et al. [57], in a system reconstituted from purified RC's and bovine heart mitochondria bc_1 complexes.

When the UQ pool is fully reduced ($E_h < 40$ mV) the oxidant produced at Q_Z must be available at Q_C in no more than 0.5–1 ms, since no lag is observed at the time resolution of our measurements under such conditions, where the first electrogenic event taking place should be the oxidation of cytochrome *b*-561. This estimate is also supported by the kinetics of cytochrome *b*-566 reduction and cytochrome *b*-561 oxidation measured at 0 mV (pH 7), in uncoupled, uninhibited chromatophores [25]. This time period does not seem to us incompatible with a true diffusive process of Q_{ox} from Q_Z to Q_C, since the oxidation at Q_Z of UQH_2 produced at the Q_B site of the RC (as evaluated from the kinetics of cytochrome *b*-561 reduction in the presence of antimycin at $E_h > 180$ mV) can occur with a lag of 800 μs [15,17,18]. A process involving two separate sites of the same protein complex can certainly occur in a comparable time period; more so if it is considered that the transmembrane diffusion of Q_{ox} could be facilitated at the protein–lipid interface of the bc_1 complex.

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