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Slow electrogenic events in the cytochrome bc_1-complex of *Rhodobacter sphaeroides*

The electron transfer between cytochrome *b* hemes can be non-electrogenic

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The flash-induced formation of transmembrane electric potential differences (measured by carotenoid bandshift) and redox changes of cytochrome b_h (b_{561}) were monitored spectrophotometrically in *Rb. sphaeroides* chromatophores in a pH range from 7.5 to 10.0. It is shown that in the presence of antimycin A and at pH < 8.3 the myxothiazol-sensitive, antimycin-insensitive component of the carotenoid bandshift is kinetically coupled to cytochrome b_h reduction. The kinetics of both processes can be described by a single exponent with a rise time of about 10 ms. Alkalization of the medium (8.3 ≤ pH ≤ 9.2) causes the appearance of an additional constituent in this phase of the carotenoid response with the rise time varying in the range of 100–300 ms. With a further pH increase (pH > 9.2), the electrogenic constituent, kinetically linked to cytochrome b_h reduction, diminishes. The obtained data are discussed within the framework of the scheme, assuming that the electron transfer between b_l and b_h hemes in the bc_1 complex is, under certain conditions, accompanied by proton transfer in the same direction.

Cytochrome bc_1 complex; Ubiquinone; Electrogenesis; Electron transfer; *Rhodobacter sphaeroides*

1. INTRODUCTION

In chromatophores of *Rb. sphaeroides*, ubiquinol molecules formed by the photosynthetic reaction center (RC) are oxidized in center Z of the cytochrome bc_1 complex (ubiquinol:cytochrome c_2-oxidoreductase), the process being accompanied by the proton release into the chromatophore interior [1]. According to the Q-cycle concept [2,3], one of the two electrons removed from ubiquinol returns via water-soluble cytochrome c_2 to the bacteriochlorophyll dimer (primary donor of RC) *P*. The other electron moves across the membrane via low-potential heme b_l (with an absorption maximum at 566 nm) and the high-potential heme b_h (with an absorption maximum at 560.5 nm) to center C (assumed to be near the outer surface of the chromatophore) where it reduces an ubiquinone molecule from the membrane pool. By monitoring the intramembrane carotenoids electrochromic bandshift [4,5] and by direct electrometry [5] two phases were identified in the formation of the transmembrane electric potential difference ($\Delta\psi$) upon oxidation of the RC-formed ubiquinol by center Z. The slower phase (the rise-time (τ) about 40 ms at pH 7.5 [5]) is suppressed by antimycin A, an inhibitor of center C activity (phase BCII). The phase remaining in the presence of antimycin A is kinetically coupled to heme b_h reduction [4,5] and is suppressed by the center Z inhibitor, myxothiazol (phase BCI). This phase is suggested to be due to the electron transfer between b_l and b_h hemes [5,6].

In the present work, the flash-induced $\Delta\psi$ formation in the bc_1 complex and redox changes of cytochrome b_h were monitored spectrophotometrically in the same samples of *Rb. sphaeroides* chromatophores in a pH range from 7.5 to 10.0. The data obtained hardly correlate with the idea of an electrogenic electron transfer between the b_l and b_h hemes.

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2. MATERIALS AND METHODS

Cells of *Rhodobacter sphaeroides* (wild type, strain R1) were grown and chromatophores were isolated by French-press treatment as in [5,7]. Absorption changes were measured and the kinetic curves were analyzed as in [5]. In Figs. 1a–3a and during the subtraction of the kinetic curves, the data were normalized according to the amplitude of phase A (see text).

During signal averaging the dark interval between the flashes was 10 s. This time is sufficient to maintain the secondary quinone acceptor (Q_B) in a semireduced form in half of the RCs before the flash (at a given concentration of redox mediators) [7]. The total amounts of flash-reducible b_h heme and of flash-oxidizable *P* were estimated from the amplitudes of absorption changes after two 5-ms-separated saturating laser flashes (two Quantel Nd lasers were used) at 561–569 nm (in the presence of antimycin A) and at 603 nm (in the presence of antimycin A and myxothiazol), respectively.

3. RESULTS AND DISCUSSION

Fig. 1a, curve 1 shows the kinetics of the electrochromic carotenoid bandshift (525 nm absorption changes) in the *Rb. sphaeroides* chromatophore suspension (pH 7.6) induced by a saturating laser flash. The fast phase (phase A), not resolvable on the time scale used, reflects charge separations in RC [1], electrogenic reduction of the photo-oxidized *P* by cytochrome c_2 [1] and protonation of doubly reduced Q_B [8] which is formed in about half of RCs after every flash [7]. The electrogenic phase which is resolvable on the millisecond time scale is due to electrogenic reactions in the bc_1 complex [5] (phase BC). Addition of antimycin A significantly suppresses this phase (Fig. 1, curve 2), further inhibition is achieved by the addition of myxothiazol (Fig. 1, curve 3). Fig. 1b shows the difference between curves 2 and 3 reflecting the electrogenic events in the bc_1 complex, which are insensitive to antimycin A, but sensitive to myxothiazol (phase BCI). The amplitude of this phase constitutes about 10% of the amplitude of phase A and the rise time coincides with the kinetics of heme b_h reduction in the presence of antimycin A (Fig. 1c; about 2/3 of the total amount of b_h heme is reduced after the flash in the pH range 7.5 to 10).

The same set of data obtained at pH 8.6 is shown in Fig. 2. The BCI phase observed at pH 8.6 (Fig. 2b) contains not only the constituent kinetically coupled to heme b_h reduction (Fig. 2c), but also an additional constituent with a hundreds ms rise time. (The appearance of the small and fast additional electrogenic constituent in the presence of antimycin A (curves 2 in Figs. 2 and 3) is due to the inhibition by antimycin A of the reverse electrogenic reaction in center C, coupled to heme b_h reduction under alkaline conditions [6,9].)

The data obtained at pH 9.45 are shown in Fig. 3. Comparison of the BCI phase (Fig. 3b) with the kinetics of the heme b_h reduction (Fig. 3c) shows that the constituent of the BCI phase kinetically coupled to heme b_h reduction is almost absent under these conditions, and that the whole electrogenesis in the presence of antimycin A is due to the electrogenic reaction with τ varying in the range of 100–300 ms.

Fig. 4 shows pH-dependences of relative amplitudes of the fast constituent kinetically coupled to heme b_h reduction (Fig. 4a), and total slow constituent (Fig. 4b) of the BCI phase (in % of phase A). Via the experimental points, the titration curves for a single proton-acceptor group are plotted. The pK values of groups in question are assumed to be 9.2 and 8.3 for the fast (Fig. 4a) and slow (Fig. 4b) constituents, respectively.

The data obtained can be explained by the following hypothetical scheme (Fig. 5).

(1) The electron transfer between the b_l and b_h hemes can be accompanied by the transfer of a proton in the

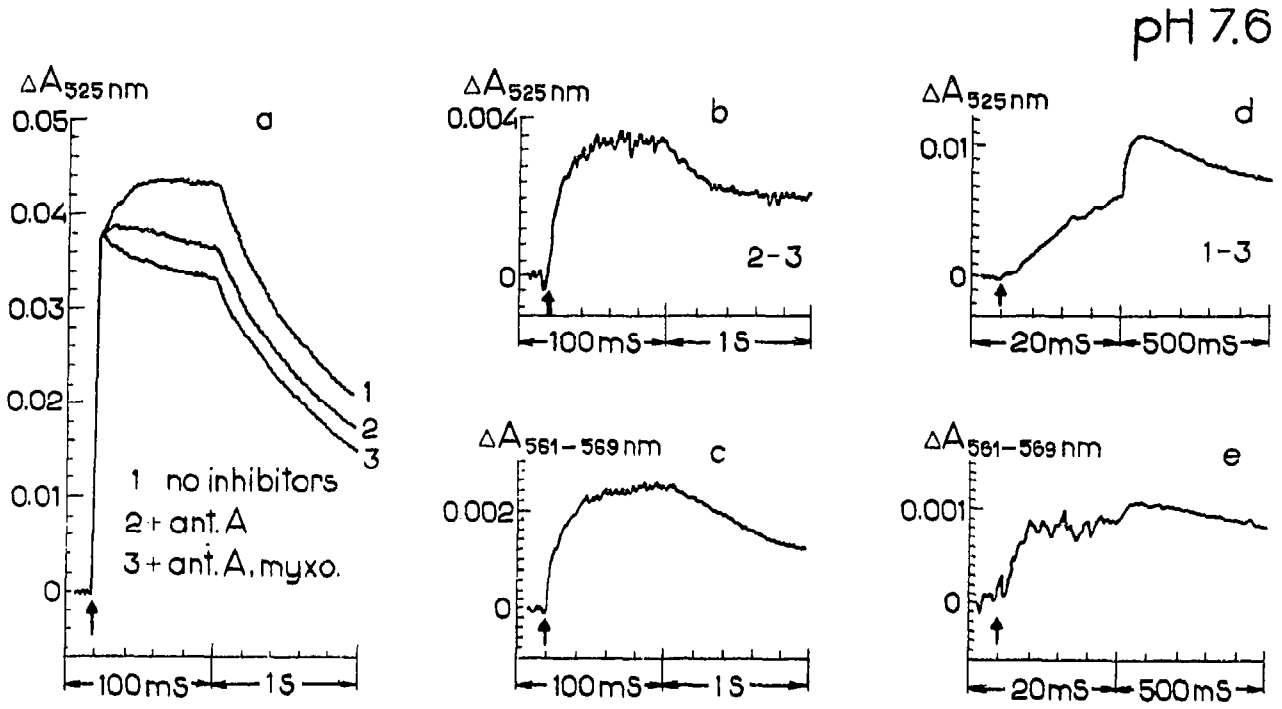

Fig. 1. Flash-induced absorbance changes in a *Rb. sphaeroides* chromatophore suspension reflecting $\Delta\psi$ formation (a,b,d) and heme b_h reduction (c,e) at pH 7.6. RCs concentration, 450 nM. Incubation medium: 20 mM HEPES, 20 mM CHES, 20 mM Bis-Tris propane and 20 mM CAPS, 5 μM TMPD (*N,N,N',N'*-tetramethyl-1,4-phenylenediamine), 2 μM PES (phenazine ethosulphate), 2 μM PMS (phenazine methosulphate), 2 mM potassium ferro/ferricyanide, 1 mM $MgCl_2$, E_h = 300 mV. Additions: 4 μM antimycin A, 5 μM myxothiazol. Arrows indicate laser flashes. Time between the flashes, 10 s. Number of averages, 25 (a,b,d), 16 (c,e).

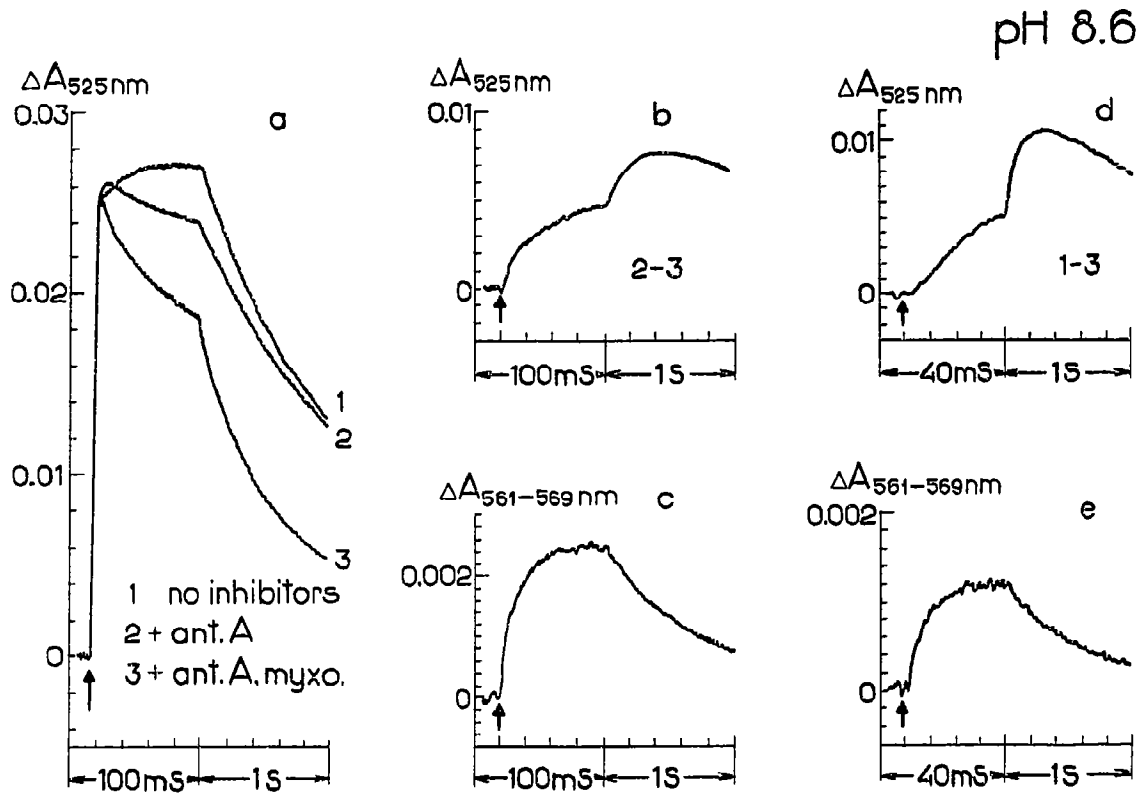

Fig. 2. Absorbance changes in *Rb. sphaeroides* chromatophore suspension reflecting $\Delta\psi$ formation (a,b,d) and heme b_H reduction (c,e) at pH 8.6. Incubation medium contained 2.5 μM TMPD, 1 μM of PMS and 1 μM of PES; other conditions as in Fig. 1.

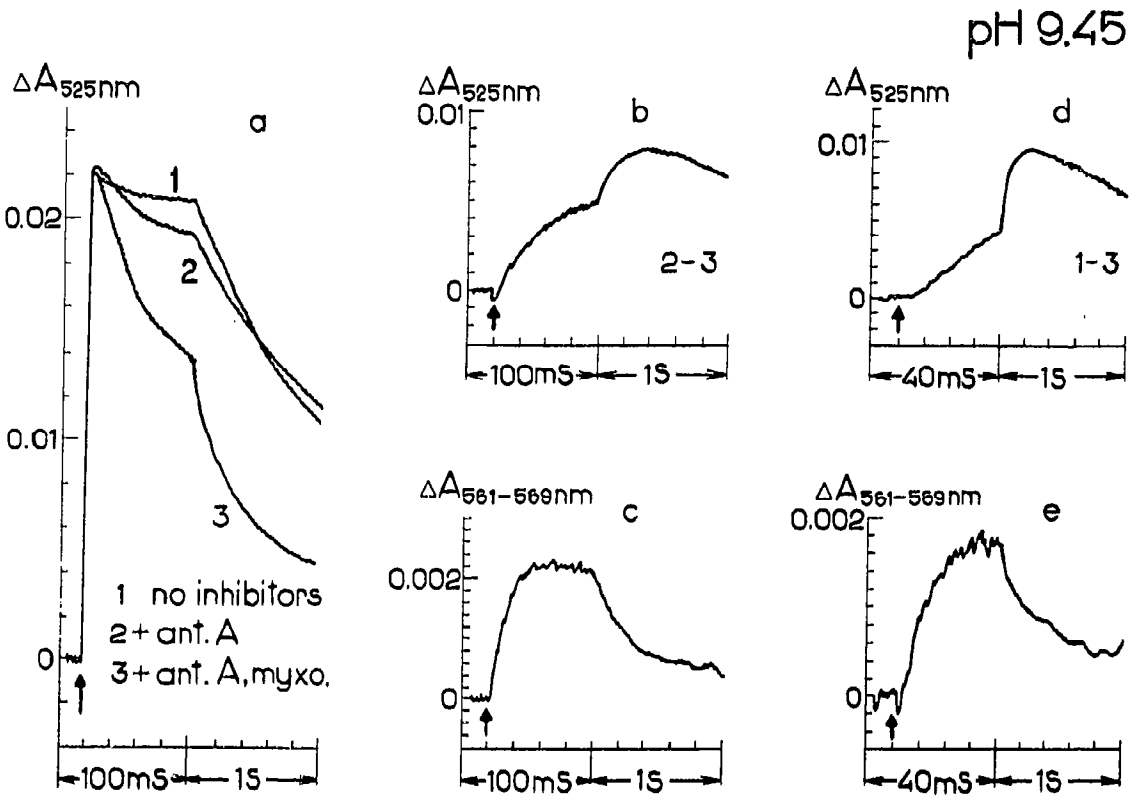

Fig. 3. Flash-induced absorbance changes in *Rb. sphaeroides* chromatophore suspension reflecting $\Delta\psi$ formation (a,b,d) and heme b_H reduction (c,e) at pH 9.45. Conditions as in Fig. 2.

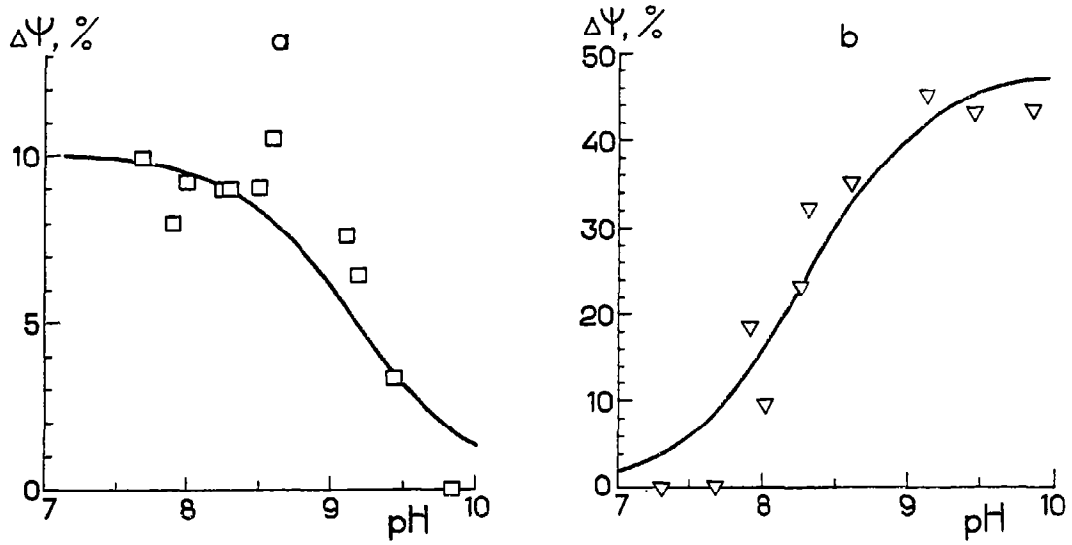

Fig. 4. pH-dependences of relative amplitudes of the BC1 phase constituents. (in % of phase A amplitude; during relative amplitudes, estimation of the relaxation of kinetical curves was taken into account. On both plates data from two independent measurements are presented). Fast, kinetically coupled to heme b_h reduction constituent (a); the total slow constituent (b). Incubation medium contained 5 μM TMPD, 2 μM PES, 2 μM PMS at pH < 8.3 and 2.5 μM TMPD, 1 μM of PMS and 1 μM of PES at pH > 8.3; other conditions as in Figs. 1-3.

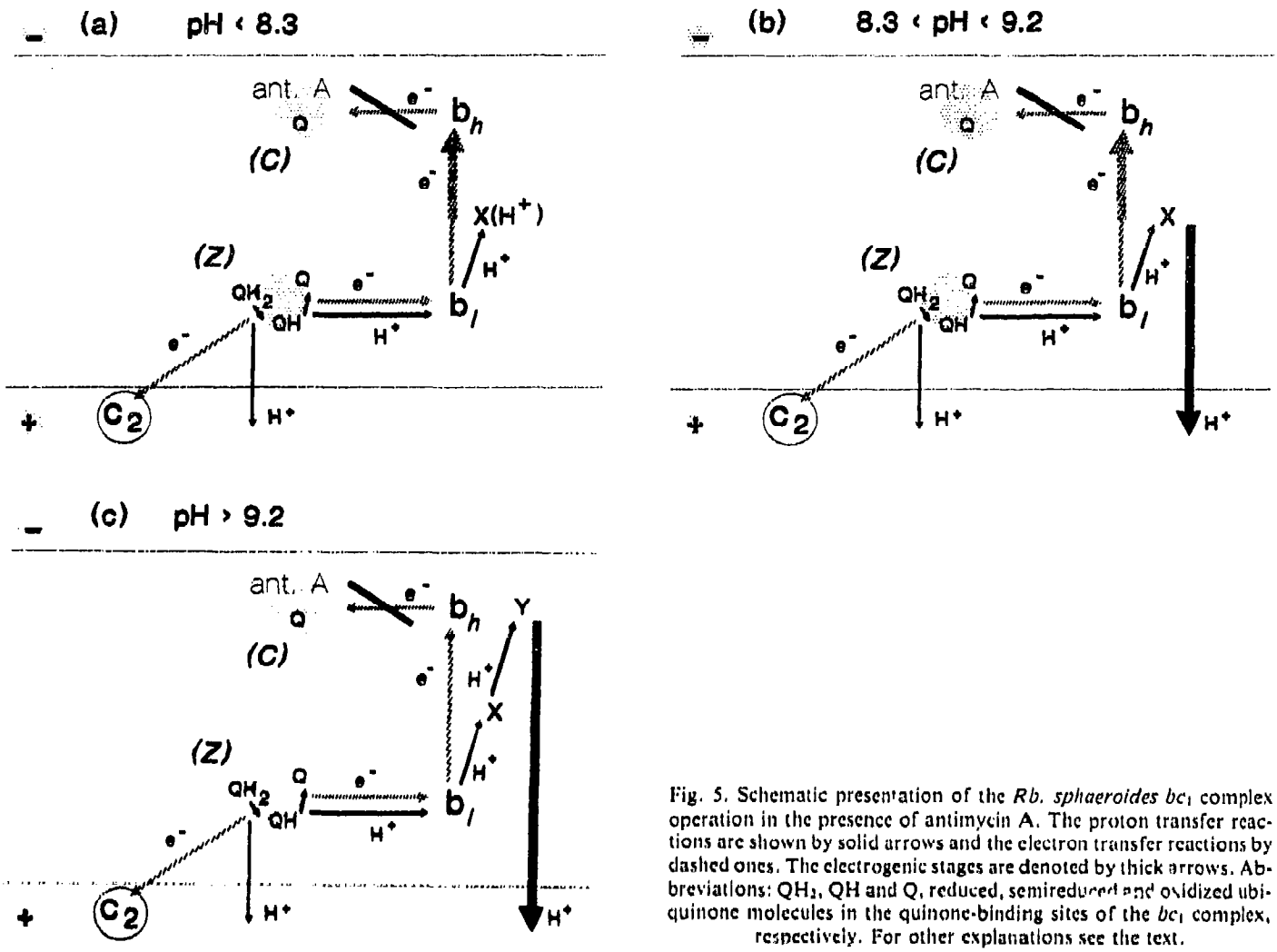

Fig. 5. Schematic presentation of the *Rb. sphaeroides* bc_1 complex operation in the presence of antimycin A. The proton transfer reactions are shown by solid arrows and the electron transfer reactions by dashed ones. The electrogenic stages are denoted by thick arrows. Abbreviations: QH_2, QH and Q, reduced, semireduced and oxidized ubiquinone molecules in the quinone-binding sites of the bc_1 complex, respectively. For other explanations see the text.

same direction under certain conditions; most probably it is one of the protons released at ubiquinol oxidation in center Z that is transported. In such a case the electron transfer between the b_l and b_h hemes is electrically silent.

(2) In the presence of antimycin A and at pH < 8.3, the proton is transferred only part of the way between the b_l and b_h hemes and protonates some group (group X) halfway between the hemes (Fig. 5a). (as it was proposed by Konstantinov [10] to explain the relatively small contribution of the BCI phase to the electrogenesis of the bc_1 complex.)

(3) At 8.3 ≤ pH ≤ 9.2 the proton does not stay at group X but is slowly released into the chromatophore interior giving rise to the $\Delta\psi$ formation, this process is observed as a slow constituent of the BCI phase (Fig. 5b).

(4) At pH > 9.2 some proton-acceptor group (Y) is deprotonated near the b_h heme and the proton transfer along the whole $b_l \Rightarrow b_h$ axis becomes possible; in this case the fast, kinetically coupled with heme b_h reduction, constituent of the BCI phase is not observed and $\Delta\psi$ is generated during the slow proton transfer to the chromatophore interior (Fig. 5c).

The above data were obtained under conditions where center C was blocked by antimycin A. It also seems interesting to consider the situation in the absence of inhibitors. Evidently, if, in this case, the electron transfer between the b_l and b_h hemes is electrogenic, the kinetics of the total electrogenic reaction in the bc_1 complex (phase BC) must contain a constituent kinetically coupled to heme b_h reduction. The kinetics of the phase BC (Figs. 1d–3d) can be determined as differences between the curves 1 and 3 in Figs. 1a–3a. The kinetics of heme b_h reduction in the same samples in the absence of inhibitors are presented in Figs. 1e–3e (b_h hemes stay reduced after the flash only if they receive an odd number of electrons; in the other case they are oxidized faster by ubiquinone than that they are reduced, see [5]). The data presented show that the kinetics of the phase BC do not contain a constituent kinetically coupled to heme b_h reduction. Thus we have to suggest that in the absence of inhibitors and at least at 7.5 < pH < 10.0 the electron is accompanied by a proton the whole way from heme b_l to heme b_h (as shown in Fig. 5c) and only then, probably after heme b_h oxidation by ubiquinone, the proton comes electrogenically into the chromatophore interior.

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