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Spectral analysis of the bc_1 complex components in situ: Beyond the traditional difference approach

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Abstract

The cytochrome (cyt) bc_1 complex (ubiquinol: cytochrome *c* oxidoreductase) is the central enzyme of mitochondrial and bacterial electron-transport chains. It is rich in prosthetic groups, many of which have significant but overlapping absorption bands in the visible spectrum. The kinetics of the cytochrome components of the bc_1 complex are traditionally followed by using the difference of absorbance changes at two or more different wavelengths. This difference-wavelength (DW) approach has been used extensively in the development and testing of the Q-cycle mechanism of the bc_1 complex in *Rhodobacter sphaeroides* chromatophores. However, the DW approach does not fully compensate for spectral interference from other components, which can significantly distort both amplitudes and kinetics. Mechanistic elaboration of cyt bc_1 turnover requires an approach that overcomes this limitation. Here, we compare the traditional DW approach to a least squares (LS) analysis of electron transport, based on newly determined difference spectra of all individual components of cyclic electron transport in chromatophores. Multiple sets of kinetic traces, measured at different wavelengths in the absence and presence of specific inhibitors, were analyzed by both LS and DW approaches. Comparison of the two methods showed that the DW approach did not adequately correct for the spectral overlap among the components, and was generally unreliable when amplitude changes for a component of interest were small. In particular, it was unable to correct for extraneous contributions to the amplitudes and kinetics of cyt b_L. From LS analysis of the chromophoric components (RC, c_{tot}, b_H and b_L), we show that while the Q-cycle *model* remains firmly grounded, quantitative reevaluation of rates, amplitudes, delays, etc., of individual components is necessary. We conclude that further exploration of mechanisms of the bc_1 complex, will require LS deconvolution for reliable measurement of the kinetics of individual components of the complex in situ.

Keywords: bc_1 complex; Q-cycle; Electron transfer; Spectral deconvolution; Kinetics; Least squares; *Rhodobacter sphaeroides*

1. Introduction

The cytochrome (cyt) bc_1 complex (ubiquinol: cytochrome *c* oxidoreductase) plays a central role in free energy transduction in biomembranes [1–5]. It has four redox centers (two hemes in cytochrome *b*, cytochrome c_1 and an iron–sulfur protein, ISP) and at least two quinone-binding sites in each monomer. The large extinction coefficients of the cytochrome components have led to a predominant use of spectroscopic methods in elucidating the mechanism of the bc_1 complex (see, e.g., [2,4,6–9]).

Purple bacteria, with their extensive photosynthetic membrane (chromatophore) system, have multiple advantages for studying the kinetics and thermodynamics of the bc_1 complex. These advantages include fast light-induced activation of bc_1 complex by its natural substrates via reaction centers, significantly different reduced-minus-oxidized spectra of low and high-potential hemes, and the convenience of well-established molecular engineering protocols. Thus, bc_1 complexes from purple bacteria are important systems for studying the structure and function of the general class of bc_1-type complexes. However, the utility of this light-induced cyclic

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; cyt, cytochrome; DM, dodecyl maltoside; DW, difference-wavelength; ISP, Rieske iron–sulfur protein; LS, least squares; RC, photosynthetic reaction center; Q_i, Q_o, quinone reducing and quinol oxidizing sites of bc_1 complex, respectively; b_L and b_H, low- and high-potential hemes of cytochrome *b*, respectively; *Rb.*, *Rhodobacter*; DAD, 2,3,5,6-tetramethyl-p-phenylenediamine

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system depends fundamentally on the ability to characterize accurately the contributions of individual components of the bc_1 complex activity.

The kinetics of the cytochrome components of the bc_1 complex in chromatophore membranes have traditionally been followed by using the difference of absorbance changes at two or more discrete wavelengths. The redox changes of the "total cytochrome *c*", c_{tot} (cyt c_1 plus cyt c_2), are usually determined at 551–542 nm [10,11]. Kinetics of cytochrome b_H are usually measured at 561–569 nm [6]. The kinetics of cyt b_L redox changes are usually followed by taking the difference in the kinetics measured at 566 and 575 nm, minus half of the changes measured at 561 minus 569 nm with further compensation for changes presumed to originate from reaction centers [12].

The traditional difference-wavelength (DW) approach tries to minimize errors due to contributions from other components of the bc_1 complex and reaction center (RC) by judicious choice of wavelength pairs. However, because of the restricted set of wavelengths used, and the simple subtractive approach, the protocols are limited in their ability to compensate for overlapping contributions, and the success of this compensation has not been established.

A large battery of different methods has been developed for the analysis of spectral data (see, e.g., [13–17]). A weakness of many of these advanced methods is the absence of a unique solution, due to the fact that absorbance changes are the (matrix) product of extinction coefficients and concentrations. Thus, without additional limitations it is impossible to find both spectra and kinetics simultaneously. The standard LS approach (sometimes called classical least-square method) takes the advantage of the knowledge of the spectra of individual components. In this case, unknown concentrations could be found from measured absorbance changes. Such approach is not based on any "hidden" models or assumptions used in more advanced approaches (see e.g. [13–17]) and can be used to recover kinetics of individual components.

Several approaches, mostly based on least-squares (LS) analysis, have been suggested to restore individual cytochrome components from overlapping spectra (see, e.g., [18–22]). In our previous work [22], we minimized this spectral interference by applying a LS analysis to kinetics measured at 10 equal steps over the wavelength range from 540 to 575 nm, using published spectra for individual components [23]. While LS analysis provides a more versatile and discriminating approach to compensating for spectral interference, its application to the characterization of kinetic changes in mutant and wild type chromatophores has been limited. In part, this is due to the lack of familiarity with the approach, and to the fact that no comparison between DW and LS analyses has been available for validation of LS analysis. In addition, it has become clear from the work reported here that the spectrum of cyt b_L previously used, while suitable for DW analysis, was not adequate for deconvolution, because of significant distortion and baseline offset [23]. This led us to recognize the need for a reinvestigation of the difference spectra for all components of the cyclic electron transport in chromatophores.

The consensus mechanism for the bc_1 complex is the Q-cycle, whose quantitative features were originally determined with the DW approach [6]. Because of the facility with which kinetics can be investigated in the chromatophores system, further development of the Q-cycle model depends heavily on the existence or development of reliable spectral analysis of these components. With this in mind, we have compared the results of the DW method with those of a least-squares (LS) deconvolution, using sets of kinetic traces at 36 different wavelengths between 540 and 575 nm, under conditions that test the accuracy of both methods. We found multiple conditions when the DW method applied to the same data sets failed to describe the characteristics of individual components with quantitative accuracy. In particular, the DW approach was generally unreliable when amplitudes of component changes were small, and especially gave incorrect amplitudes and kinetics for cyt b_L. Inaccuracies in previous determinations of the kinetics of individual components are discussed as indicating a need for reevaluation of some quantitative properties of the model (halftimes, amplitudes, delays, etc.). Such reevaluation is especially important when analysis of the bc_1 complex function is done under conditions (pH, temperature, E_h, inhibitors, multiple flashes, mutant strains), for which changes of the species of interest are relatively small and other components with weaker absorbance at the wavelengths used can contribute significantly. These are often the conditions necessary for a deeper understanding of mechanism, and for successful prediction of the behavior of the bc_1 complex under operating conditions.

2. Materials and methods

2.1. Growth of cells and isolation of chromatophores

Cells of the wild type *Rb. sphaeroides* Ga were grown photosynthetically at 30 °C in Sistrom's medium. Cells of GBH6 strain of *Rb. sphaeroides* having the His-tag at the C terminus of the cyt *b* subunit were grown photosynthetically at 30 °C on Sistrom's medium supplemented with 2 μg/ml of tetracycline and 10 μg/ml of kanamycin [24]. Chromatophores were isolated by differential centrifugation as described elsewhere [25].

Cells of the pC2P404.1 strain (native cyt c_2 superproducer) of *Rb. sphaeroides* Ga [26] were grown photosynthetically at 30 °C in Sistrom's medium in the presence of 2 μg/ml tetracycline. This strain was used in some experiments to test the ability to compensate for the large changes in absorbance due to the excess cyt c_2.

2.2. Spectrophotometric measurements

Absorption spectra were measured using an Agilent 8453 diode array spectrophotometer.

Kinetics of cytochromes and the electrochromic carotenoid bandshift were measured with single beam kinetic spectrophotometers of local design. Light pulses were provided by a xenon flash (~5 μs half-width).

The electrogenic activity of the photosynthetic chain was monitored at 503 nm through the carotenoid electrochromic bandshift. Under the conditions used in all kinetic experiments (5 μM gramicidin, 25 μM CCCP) the amplitude of the electrochromic change was reduced by 80%, due to a fast unresolved decay; the remaining amplitude decayed in ~0.5 ms. However, the amplitude of electrochromic changes in the α-band region was small and did not contribute significantly to the kinetic traces used to determine the redox changes of individual components. In critical cases, we checked this by repeating experiments at 10-fold higher gramicidin concentrations.

The monochromator (Triax 180 (JobinYvon), 1200 groves/mm grating) was calibrated using Hg-vapor lamp emission bands in the α-band region. The wavelength resolution was 0.3 nm, and reproducibility was 0.06 nm. The slit width used in kinetic measurements was set at 0.5 mm to give a bandwidth of 1.8 nm.

2.3. Least squares method

The least squares (LS) method minimizes the sum of the squares of the deviations of experimentally measured values of absorbance changes from a theoretical expectation (see, e.g., [14–16,18–22]). The LS method is based on the representation of absorbance changes at each wavelength, as the sum of the absorbance changes of each individual component, assuming that the absorbance of each component obeys the Beer–Lambert Law. Because the spectra used are scaled in terms of extinction coefficients, and the traces used as input are scaled in absorbance units, the output of the LS fit is also in absorbance units.

Absorbance changes at wavelength λ_i ($i=1,\ldots,L$) is the sum of the absorbance changes for each individual component j, $j=1,\ldots,N$, present in the system:

$$A(k_i) = \sum_{j=1}^{N} A_j(k_i) \tag{1}$$

in turn, it is assumed that absorbance of each j-th component obeys the Beer–Lambert Law, i.e., the optical density of unit path length is the product of the millimolar extinction coefficient s_j (with units of mM^{-1} cm^{-1}) at each wavelength and the concentration c_j (in mM) of each component in solution:

$$A_j(k_i) = s_j(k_i)c_j. \tag{2}$$

Combining Eqs. (1) and (2), we have:

$$A(k_i, t_k) = \sum_{j}^{N} s_j(k_i)c_j(t_k) \tag{3}$$

we assumed that only concentrations of individual components depend on the time, i.e., $c_j = c_j(t)$.

In matrix notations, this can be presented as follows:

$$A = SC \tag{4}$$

where $A = \{a_{ik}\} \equiv A(\lambda_i, t_k)$, $(i = 1,\ldots,L)$; $S = \{s_{ij}\} \equiv s_j(\lambda_i)$, $(i = 1,\ldots,L; j = 1,\ldots,N)$; $(C = \{c_{jk}\} \equiv c_j(t_k)$, $(j = 1,\ldots,N)$.

The LS method minimizes the sum of the squares of the deviations of experimentally measured values of absorbance changes from the theoretical one given by Eq. (4), i.e. it minimizes $EE^{T} = (A-SC)(A-SC)^{T}$ (here superscript 'T' indicates the transpose of a matrix).

Under wide conditions, the matrix

$$\bar{C} = (S^T S)^{-1} S^T A \tag{5}$$

gives the best estimate of the unknown matrix C from known matrix S and measured matrix A in Eq. (4) [22,23]. Similarly, the matrix

$$\bar{S} = AC^T (CC^T)^{-1} \tag{6}$$

gives the best estimate of the unknown matrix S from known matrix C and measured matrix A.

2.4. Isolation of bc_1 complex

Cytochrome bc_1 complex was isolated from chromatophores prepared from *Rb. sphaeroides* strain GBH6 and purified in a single step by following the protocol described by Guergova-Kuras et al. [24] with two main modifications: (i) MOPS at each step was replaced by Tris–HCl, pH 7.4 (25 °C) and (ii) azolectin (Sigma-Aldrich, St. Louis, MO) and *E. coli* total lipid extract (Avanti polar lipids, Alabaster, AL) were used as sources of phospholipids instead of phosphatidylcholine. The purified enzyme was concentrated using Amicon centrifugal filter concentrators (Millipore Corporation, Bedford, MA) with a cutoff of 10,000 Da at 4 °C. The concentration of cytochrome bc_1 complex was estimated from the ascorbate-reduced minus ferricyanide-oxidized difference spectrum of cyt c_1 assuming an extinction coefficient of 20 mM^{-1} cm^{-1} at 552–542 nm.

2.5. Reagents

Antimycin A, azolectin, gramicidin, myxothiazol, stigmatellin, DAD, PMS, CCCP, MOPS and Tris were obtained from Sigma-Aldrich (St. Louis, MO). Dodecyl maltoside (DM) was purchased from Anatrace (Maumee, OH). Inhibitors and uncouplers were dissolved in ethanol and stored at −20 °C.

3. Results

3.1. Spectra of individual components of the bc_1 complex

The application of classic least squares analysis requires knowledge of the individual spectra for each component. As this work shows, these critical data are not well defined, either for the isolated bc_1 complex or for chromatophores, and we first reexamine them, here.

3.1.1. Spectra for the isolated bc_1 complex

The reduced minus oxidized spectra of individual components in isolated bc_1 complexes from *Rb. sphaeroides* are shown in Fig. 1. The difference spectrum of cytochrome c_1 has a single α-band with maximum at 552.5 nm and maximum, originating from the shift of the γ band, at 420.3 nm (as determined by fitting the top of each band with a single Lorentzian). The difference spectrum of b_H heme has an α-band at 560.5 nm and the maximum of the shift of the γ band at 429.3 nm. The difference spectrum of b_L heme has a double α-band with

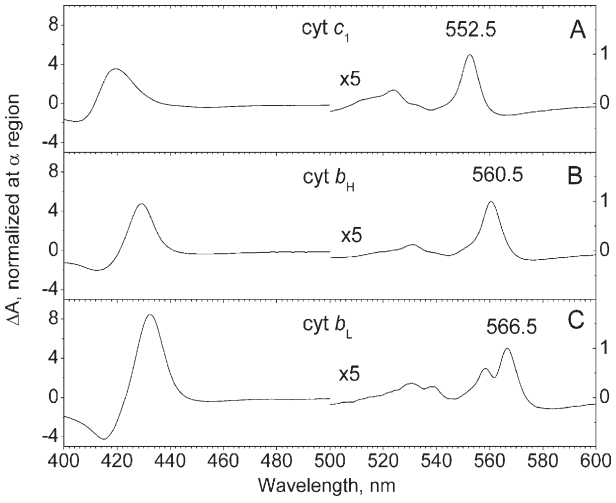

Fig. 1. Reduced-minus-oxidized difference spectra of cytochrome components in the bc_1 complex from *Rb. sphaeroides*. (A) Ascorbate-reduced minus ferricyanide-oxidized spectrum of the bc_1 complex. (B and C) Dithionite-reduced minus ascorbate-reduced spectra of the bc_1 complex, measured immediately after dithionite addition (B), or (C) the difference after a further 5-min incubation. Medium in panel A: 50 mM Tris–HC1, pH 7.4, 100 mM NaCl, 1 mM $MgSO_4$, 0.01% DM. Medium in panels B and C: 25 mM Tris–HC1, pH 7.4, 75 mM NaCl, 0.5 mM $MgSO_4$, 0.01% DM, 10% glycerol, 1 mM Na-ascorbate. Concentration of bc_1 complex is 1.9 μM in panel A and 1.7 μM in panels B and C. All spectra are normalized to an absorbance of 1 at the maxima of the main α bands.

maxima at 566.5 and 558.5 nm and the maximum of the shift of the γ band at 432.7 nm.

3.1.2. Difference spectra of individual components in chromatophores in the 540–575 nm region

Reduced-minus-oxidized spectra of individual components in *Rb. sphaeroides* chromatophores in the 540–575 nm range were originally obtained by separating individual components during redox titration [23]. In general, those spectra correspond to the spectra we observed in this work. However, the spectra for c_1 and b_L hemes were significantly distorted in the original work, especially by baseline shifts. Our initial corrections utilized the general shape of the cytochrome components in the isolated bc_1 complex shown in Fig. 1. Further corrections of the spectra were achieved by comparing the input spectra with those reconstructed from the deconvolution of kinetic changes using Eq. (6). To compare the efficacy of the traditional DW and LS methods under equivalent conditions, we have measured and deconvoluted kinetic data in samples of chromatophores at different redox potentials, and in the presence of specific inhibitors of the bc_1 complex, such as antimycin A, myxothiazol, and stigmatellin. Altogether, we analyzed over 20 different data sets with at least 700,000 points in each set. Fig. 2 shows the spectra used in this paper.

The spectra shown in Fig. 2 were also normalized according to published values of the extinction coefficients. For cyt c_2 we took $\Delta\varepsilon(551–542\ \text{nm}) = 20\ \text{mM}^{-1}\ \text{cm}^{-1}$ [10]. The extinction of cytochrome b_H at 561–569 nm was taken as $20\ \text{mM}^{-1}\ \text{cm}^{-1}$ [6]. The extinction coefficient for P870 at 542 nm was assumed to be $10\ \text{mM}^{-1}\ \text{cm}^{-1}$ [27]. The extinction coefficient for cyt c_1 was taken from its value, relative to cyt c_2, seen in chromatophores in the presence of antimycin alone.

Fig. 2 allows simple estimation of the best-case uncertainty in the traditional difference-wavelength analysis when the concentration changes of individual components are similar. For example, the difference extinction coefficients at 561–569 nm for cyts b_H, and c_1 are ~20, and ~3, respectively. Thus, for identical concentration changes in each species, ~87% of absorbance changes at 561–569 nm would correspond to b_H heme, but ~13% of the absorbance changes would originate from cyt c_1. When the change in b_H is smaller than that of c_1, the correspondence is worse, leading to significant distortions, for example, in the early phases of the kinetics. The situation for b_L heme is more complicated (see below).

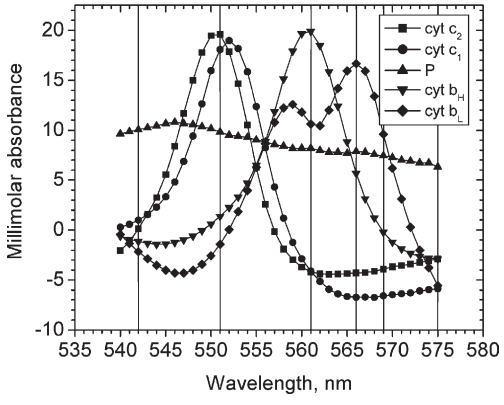

Fig. 2. Difference spectra of individual components in the 540–575 nm region in *Rb. sphaeroides* chromatophores. Difference spectra of cyt c_2, cyt b_H and P870 were determined by Meinhardt [23]. The spectra for cyt c_1 and cyt b_L were corrected and refined as described in the text. Vertical lines mark the "traditional" wavelengths usually used for the determination of individual components in chromatophores.

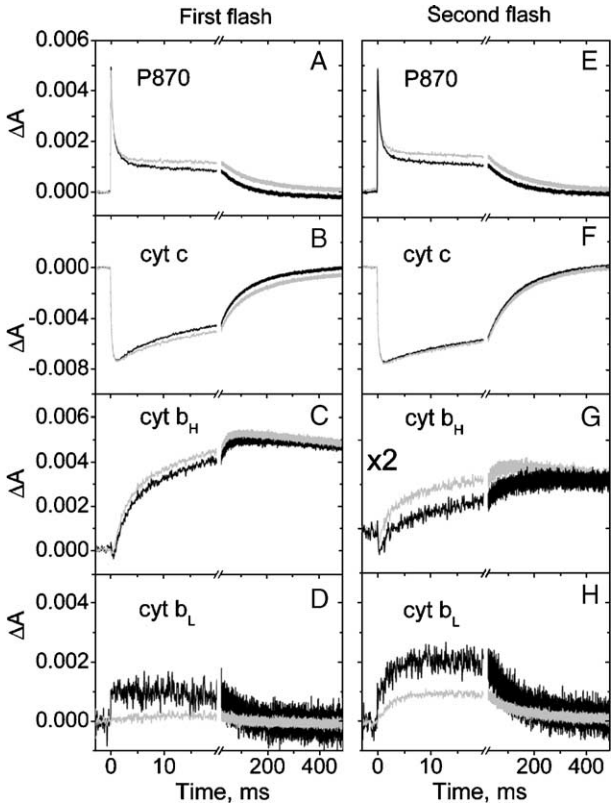

Fig. 3. Flash-induced kinetics of individual components of *Rb. sphaeroides* chromatophores in the presence of antimycin. The kinetics were determined using the traditional DW approach (black), and by LS analysis using kinetic traces measured at 36 wavelengths from 540 to 575 nm, with 1 nm step (gray). (A–D) Changes induced by the first flash; (E–H) changes induced by the second flash. Note that the scale in panel G is different from that in panel C. Chromatophores were suspended in 50 mM Tris–HCl buffer (pH 7.4) with 10 mM potassium ferrocyanide, 100 μM DAD, 2 mM NaCN, 25 μM CCCP, 5 μM gramicidin, 5 μM antimycin A. Kinetic traces at each wavelength are the average of 2 traces, with 30 s between measurements and were recorded with an instrument response time of 50 μs. The redox potential was ≈280 mV.

3.2. Kinetics of individual components determined using DW and LS approaches

3.2.1. Kinetics at 280 mV

At ambient redox potential E_h ~280 mV, P870 is completely reduced, cyt c_2 is 90% reduced, the high-potential components of the bc_1 complex (cyt c_1, E_{m7} = 260 mV, ISP, E_{m7} = 315 mV) are partially reduced, while cyt b_L (E_{m7} = −90 mV), cyt b_H (E_{m7} = +50 mV) and the ubiquinone pool (E_{m7} = 90 mV) are fully oxidized. Flash activation of the RC under these conditions leads to the generation of oxidized cyt c_2 and QH_2, which are the substrates for the bc_1 complex.

Fig. 3 shows first and second flash-induced kinetics of four components in *Rb. sphaeroides* chromatophores in the presence

of antimycin A, an inhibitor of the Q_i site. For the DW analysis, the redox changes of each component were assessed as follows: cyt c_1 plus cyt c_2 (=c_{tot}) were followed at 551–542 nm [10,11]; cyt b_H reduction was measured at 561–569 nm [6]; cyt b_L redox changes were measured by taking the difference in the kinetics measured at 566 and 575 nm, minus 1/2 of the changes measured at 561 minus 569 nm [12], but the suggested compensation for P870 was omitted because our analysis showed that the spectral interference was an overlap of contributions from P870 and both cyt *c* hemes. These will be discussed more fully in a separate paper. The kinetics of P870 were measured at 542 nm [27]. The kinetics determined by the DW method are shown in black; those obtained by LS deconvolution in gray. The deconvolution was based on the spectra in Fig. 2, and kinetic traces were measured at 36 equally spaced wavelengths between 540 and 575 nm, including those used in the DW approach. Comparison of the kinetics induced by the first flash indicates quantitative differences (Fig. 3A–D):

(1) Although the overall kinetics of $P870^+$ are similar, the amplitude remaining after rapid rereduction is significantly (20–25%) larger when determined via deconvolution than from the trace measured at 542 nm. The kinetics measured at 542 nm also show a negative change at $t > 0.2$ s, which would not be possible with P870 initially completely reduced, while the P870 kinetics determined via LS deconvolution are always positive, as expected.
(2) The fast kinetics of cyt c_{tot} are practically identical when determined by both DW and LS analyses. However, differences are apparent in the rereduction phase at longer times.
(3) The delay before onset of cyt b_H reduction after the first flash, measured by extrapolation to the zero line, is half as long (400 μs vs. 800 μs) when cyt b_H kinetics are determined by LS deconvolution. The difference reflects mainly a downward deflection in the DW kinetics that is clearly artifactual.
(4) The concentration of b_L heme determined by LS deconvolution is ca. 4-fold smaller than the concentration determined by using the 4-wavelength difference procedure, and does not show a rapid step-change immediately after the flash. The noise level in the LS deconvolution is significantly less, mostly due to the effective averaging of the large number of wavelengths used for the deconvolution.

The kinetic changes induced by the second flash show the following differences when determined by the DW and LS methods (Fig. 3E–H):

(1) The amplitude of the $P870^+$ change determined via deconvolution is again larger than that determined from the single wavelength measurement at 542 nm.
(2) The kinetics of cyt c_{tot} are practically identical in both DW and LS analyses, over the whole time course. This is because the DW method works well for c_{tot} in the absence of significant changes in the *b*-hemes—the interference from b_H on the first flash is clear in Fig. 3B.
(3) The kinetics of cyt b_H induced by the second flash differ significantly when determined by DW or LS analyses. In particular, the DW trace shows a negative "dip" (apparent oxidation) and markedly polyphasic kinetics. The kinetics of reduction in the LS deconvolution are more nearly monophasic. The relative difference in amplitudes yielded by the two methods is many-fold at short times. In previous analyses, changes in this time range were recognized as artifactual and ignored. Nevertheless, the DW trace is still 2 times smaller at 20 ms.
(4) The amplitude of cyt b_L reduction determined by LS deconvolution is substantially smaller than that determined by the DW method, largely due to an initial step-change in the latter case (see above). When determined by LS deconvolution, a delay of ~0.4 ms can be seen in the kinetics of cyt b_L, followed by a smooth, monophasic kinetic.

The larger relative difference in cyt b_H kinetics determined by DW and LS analyses for the second flash is an excellent example of the importance of proper decomposition of the spectral interference among components. In the presence of antimycin, the first actinic flash leads to almost complete reduction of cyt b_H. The second flash therefore induces significantly less amplitude and, as a result, other absorbing species have a greater impact. Under these circumstances, the DW analysis "confuses" cyt b_H with cyt c_{tot} and possibly with P870, while LS analysis minimizes such spectral interference. It is important to stress that in all cases described in this work, both analyses were based on the same set of kinetic data measured, in this case at 36 different wavelengths between 540 and 575 nm. Thus, the differences between these analyses are due to the ability of the particular method to recover the correct kinetics using different sets of wavelengths.

Fig. 4 shows the flash-induced kinetics of the same four components in chromatophores in the presence of both antimycin A and myxothiazol (inhibitors of the Q_i and Q_o sites, respectively). Since both pathways for access of redox equivalents are blocked, and both *b*-hemes were initially oxidized, all redox changes of the b-hemes should be eliminated. This therefore provides a good test of the deconvolution procedure. The kinetics of the components were determined using DW analysis (black) and LS deconvolution (light gray). Comparison of the kinetics of different components determined by DW and LS analyses shows quantitative differences, considered below.

The quasi-stationary amplitude of the $P870^+$ change determined by LS analysis is identical to that determined at 542 nm. However, significant differences are apparent in the rereduction phase at longer times. Kinetics of cyt c_{tot} determined by DW and LS analyses are practically identical because the b-hemes do not change. On the other hand, in spite of the expected blockage of electron access to the *b*-hemes, the traditional DW method shows substantial, apparent flash-induced oxidation of cyt b_H. LS deconvolution also revealed some apparent redox state changes in cyt b_H, which, however, is within the uncertainty of the b_H spectrum. For cyt b_L, no redox

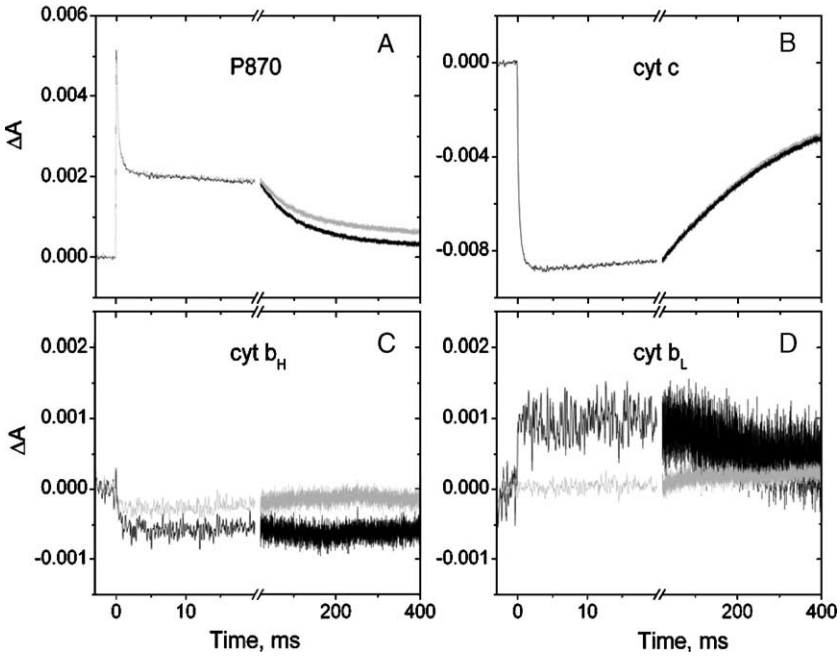

Fig. 4. First-flash-induced kinetics of individual components of *Rb. sphaeroides* chromatophores in the presence of antimycin and myxothiazol. The kinetics were determined using the traditional DW approach (black), and by LS analysis using kinetic traces measured at 36 wavelengths from 540 to 575 nm, with 1 nm step (light gray). Chromatophores were suspended in 50 mM Tris–HCl buffer (pH 7.4) with 10 mM potassium ferrocyanide, 100 μM DAD, 2 mM NaCN, 25 μM CCCP, 5 μM gramicidin. Antimycin A and myxothiazol were present at concentrations 5 μM each. Kinetic traces at each wavelength are the average of 2 traces, with 30 s between measurements and were recorded with an instrument response time of 50 μs. The redox potential was ≈280 mV.

change was seen when determined by LS analysis, in contrast to a significant step-change seen with the DW analysis. The change in b_L seen in the DW analysis is larger than expected from the uncertainty of the b_L heme difference spectrum in Fig. 2. Because there should be no electron transfer to or from the *b* hemes in the presence of antimycin and myxothiazol, we assume that any changes seen, especially in the DW analysis, are actually due to P870 or *c*-cytochrome (see Fig. 2). For the same reason, such changes were ignored in previous analyses.

3.2.2. Kinetics at 150 mV in the presence of antimycin A

At ambient redox potential E_h ~150 mV, all high-potential components of bc_1 complex (cyts c_1 and c_2, and ISP) are fully reduced, while low-potential components of bc_1 complex, cyt b_L (E_{m7} = −90 mV) and cyt b_H (E_{m7} = +50 mV) are oxidized, and the ubiquinone pool contains ~1 QH_2/bc_1 complex. Under these conditions, the flash induced activation of bc_1 complex is limited both by the delivery of oxidizing equivalents from RC and by the low concentration of reductant in the membrane.

Fig. 5 shows the flash-induced kinetics of the four components in chromatophores prepared from the cyt c_2 "superproducer" strain of *Rb. sphaeroides* at E_h ~150 mV in the presence of antimycin A, determined using traditional DW (black) and LS (gray) analyses. The excess of cyt c_2 provides a test of the success in deconvoluting this component from the kinetics of all the others.

The kinetics of cyt c_{tot} and cyt b_H look very similar and coincide within 20% for both DW and LS analyses. However, the amplitudes and kinetics of P870 and cyt b_L are significantly different. This difference arises from the fact that the changes in concentration of the main absorbing components are small, so that other absorbing species begin to play more significant roles. In the cyt c_2 superproducer the concentration of "stable" oxidized P870 pigment is smaller than in the wild type (see Figs. 3 and 4) due to faster, more effective, reduction of pigment by cyt c_2. In this situation small negative changes at 542 nm introduced by the cytochrome components have a significant impact on the observed signal. Thus, the quasi-stationary level of $P870^+$ after each flash is underestimated by the DW method and is at least 2-times larger when determined by LS deconvolution.

The kinetics and amplitude of cyt b_L reduction are even qualitatively different in the two analyses. On the first flash, the DW method yields a fast step-change in cyt b_L, well recognized as artifactual; subsequent flashes show a similar step-change followed by poorly defined further changes. With the LS analysis, the first flash shows a small but time-resolvable reduction, and subsequent flashes show a steady accumulation of reduced b_L with similar kinetics.

4. Discussion

4.1. Difference spectra of individual components in isolated bc_1 complexes and in chromatophores

In order to apply the LS deconvolution, we found it necessary to invest considerable effort in correction of the spectra of individual components. First, we measured spectra of all cytochrome components in isolated bc_1 complex. The true zero lines in these difference spectra were found from the region near 750 nm where we do not see any background absorbance. Such corrected spectra are shown in Fig. 1. We used these

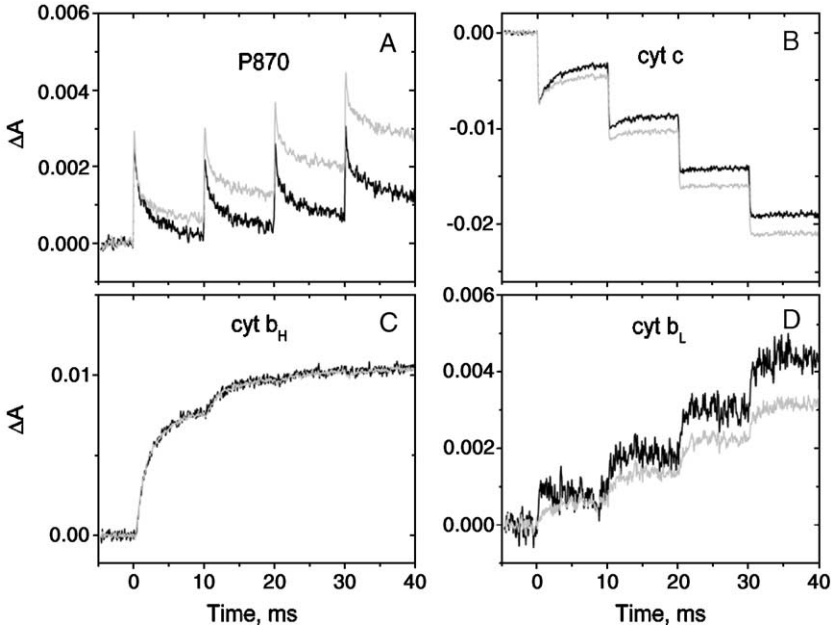

Fig. 5. Kinetic traces of individual components on flash excitation of *Rb. sphaeroides* cyt c_2 superproducer chromatophores at 150 mV in the presence of antimycin alone. The kinetics were determined using the traditional difference-wavelength approach (black), and by LS analysis based on the use of kinetic traces at 20 wavelengths measured between 540 and 576 nm (light gray). Chromatophores of cyt c_2 superproducer were suspended in 10 mM MOPS buffer (pH 7), 100 mM KCl with 4 μM PMS, 1 mM Fe–EDTA, 300 μM Na ascorbate, 1 mM KCN, 10 μM antimycin A, 25 μM CCCP, and 5 μM gramicidin. Kinetic traces at each wavelength are the average of 8 traces, with 20 s between measurements and were recorded with an instrument response time of 33 μs. The redox potential was +150 mV (±30 mV).

spectra as a guideline for difference spectra for cytochrome components of the bc_1 complex in situ in the chromatophores membrane (Fig. 2). From inspection of Fig. 2, it will be apparent that the adjustment of baselines does not affect the DW approach, but is crucial for the LS analysis. Finally we used Eq. (6) to tune the spectra to self-consistency, within approximately 5% error, by comparing results of deconvolution of kinetic changes measured in chromatophores at different conditions.

We re-measured difference spectra in chromatophores for all the main absorbing species in the region 540–575 nm. We found that the original spectra obtained by Meinhardt [23] via redox titration were generally correct in shape, but exhibited significant baseline offsets that were substantial for cyt b_L and cyt c_1. This is critical for application of a LS analysis, as the magnitudes of the absorbance changes are required rather than just differences.

Each spectrum shown in Fig. 2 is an average representation of the multiple experiments obtained for isolated bc_1 complex and chromatophores. The reasonable correspondence of all these approaches significantly adds to our confidence in the general shape of individual spectra. The deviation between different methods (after vertical shift and normalization) is within 8% for b_L heme and P870 and within 5% for all other components.

4.1.1. Spectrum of b_L heme

The spectrum of b_L heme in the isolated bc_1 complexes and in chromatophores from *Rb. sphaeroides* has two well-resolved peaks at ~558 and ~566 nm (Figs. 1 and 2). These spectra correspond well to the spectra obtained by Meinhardt and Crofts ([12], Fig. 1) after vertical shift and normalization of latter.

The difference spectrum of b_L heme in mouse bc_1 also has two distinct peaks [28]. However, only a shoulder is seen in bovine [29–31], and yeast [32] complex III. Thus, we might conclude that the splitting of alpha band of b_L heme is species-dependent. It is clear that position, half-widths, relative amplitude of two bands of b_L heme and difference of midpoint potentials between b_L and b_H are all important for the ability to see either shoulder or two well-separated maxima.

The splitting of the alpha-band for b_L heme is similar to the splitting observed in the absorption spectra of many other low-spin ferrous hemoproteins [29,30,33–37]. Different factors, such as internal motion of the heme group, axial ligands, peripheral substituents, and electrostatic field could be responsible for the spectral splitting of the Q-band [34–37].

The relative extinction coefficients for cyt *b* hemes reported in this paper (Fig. 2) are similar to those determined previously by redox titration of flash-induced measurements of b_L and b_H hemes in *Rb. sphaeroides* chromatophores, where the peak of b_L heme is ~0.8 of that for the peak of b_H heme [12,38]. Our current value, ~0.83 (Fig. 2), is derived from the analysis of spectral and kinetic information of many multi-flash experiments in *Rb. sphaeroides* chromatophores under different conditions. Close values for extinction coefficients of b_L and b_H hemes were also reported for other species. For example, Howell and Robertson [28] reported ratio of extinction coefficients ~0.72 for bc_1 from mouse cells. However, in some cases, the extinction coefficient for b_L heme was reported to be less than that for b_H heme by a factor of 2, or even 3 (see, e.g., [39]). The reasons for this discrepancy are not clear, and could include species-specificity but also inability to properly separate spectral changes of b_H and b_L hemes.

4.1.2. Spectrum of b_H heme

Our spectrum for b_H heme is in good correspondence with previous measurements [12,23]. The difference between the normalized spectrum for b_H heme shown in Fig. 2 and those obtained by Meinhardt [23] does not exceed 5%. However, the band is subtly asymmetrical and raises the question whether some minor heme *c* component is present on the low wavelength side of heme b_H difference spectrum. We see this asymmetry in all spectra we obtained, both for isolated bc_1 and chromatophores, and it is present in spectra obtained by Meinhardt and Crofts [12] during redox titration of chromatophores. This feature is also seen in flash-induced spectra in chromatophores. Deconvolution of the spectrum of b_H heme (Fig. 2) into two Gaussians did not reveal the presence of a 552 nm band, and, if the center of one of two bands was fixed at 552 nm during the fitting, the width of this band was >30 nm. This is very much greater than expected for any cytochrome component. In addition, in isolated bc_1 complexes we were unable to see any significant contribution from cyt c_1 in the gamma band. Thus, we conclude that the "foot" observed on the low wavelength side of heme b_H difference spectrum is not certainly due to cyt c_1 alone, and should probably be considered to correspond to the b_H heme, too. Nevertheless, the possibility of a small contamination from a low potential form of c_1 is difficult to exclude [40–43]. In any event, the extinction coefficient in this region is close to zero and is well within the 5% uncertainty of the peak spectra shown in Fig. 2. It therefore does not pose a significant problem for the deconvolution.

4.2. Comparison of least squares (LS) and difference-wavelengths (DW) methods

The ability to resolve spectra of low and high potential hemes of cyt *b* in chromatophores of non-sulfur purple bacteria was a significant factor facilitating the development of the modified Q-cycle model [1,6,7]. Moreover, the testing of molecular mechanisms of bc_1 turnover depends heavily on the simultaneous analysis of all components in the system.

To characterize the kinetics of cyclic electron transport in *Rb. sphaeroides* chromatophores we adapted a least squares analysis. This method provides a natural way of deconvoluting overlapping spectral bands when the spectra of individual components are known. It is therefore important to stress that the method depends on the assumption that no other absorbing species contribute to the observed changes. The two most significant interferences in chromatophores are the electrochromic bandshift of carotenoids [44–47] and bacteriochlorophylls [48,49], and the presence of highly absorbing redox mediators. These factors were minimized in this paper by adding uncouplers to eliminate electrochromic bandshift and by using weakly absorbing redox mediators.

The difference-wavelength analysis [6,10,12] is very simple and in many cases provides an adequate description of electron transfer in the bc_1 complex (see Figs. 3–5). In particular, the large amplitude kinetics of cyt c_{tot} and heme b_H, which formed the main experimental support for the modified Q-cycle, are not significantly different from those generated by the more complete LS analysis. However, smaller amplitude signals and, especially, the kinetics of heme b_L determined by DW analysis are significantly different from those determined by the LS analysis. Meinhardt and Crofts [12] suggested an ad hoc correction based on the $P870^+$ amplitude, which was fairly successful at eliminating the fast step-change in b_L but, as described above, the artifact actually includes contributions from the *c*-cytochromes, as well.

It is clear that the traditional difference-wavelength analysis can give significant errors when the absorption changes due to the component of interest are small, and, as a result, the input of other species becomes significant. A typical example would be the activation of the bc_1 complex via QH_2 oxidation at the Q_i site, observed in the presence of myxothiazol. Under these conditions the flash-induced changes of cyt b_H are small and the contribution from cyt c_1 becomes significant when measured at 561–569 nm.

In the general case, one would prefer to use the spectra of cyt b_H and b_L measured in the presence of different inhibitors (antimycin, myxothiazol and others) because the inhibitors induce small shifts in the spectra of the respective hemes (see, e.g., [28,50,51]). However, in most cases, the shifts are quite small (≤0.5 nm) and to a good approximation we can ignore these effects. The traditional DW approach does not distinguish between these cases, and in the current LS analysis, we also have not used separate spectra for different inhibitors. It should be noted, however, that the corrected spectra we use (Fig. 2) are derived from multiple conditions, including the presence and absence of inhibitors, and they therefore represent averages over the various conditions.

We expect that future analyses will utilize spectra of b_H and b_L hemes determined for specific conditions such as in the presence of different inhibitors. However, it is also possible that the detailed spectra of some components depend on the redox state of others (especially hemes b_L and b_H) or on membrane potential. If so, there will be no unique spectral basis set, and self-consistency will be the only benchmark.

4.3. The need for LS analysis based on smaller wavelength sets

To determine the kinetics of individual components in *Rb. sphaeroides* chromatophores we measured kinetic traces at 36 wavelengths between 540 and 575 nm. This allowed the iterative restoration of the component spectra (see Eq. (6)), and very high accuracy in the deconvolutions, and included all wavelengths traditionally employed by the DW method. However, such coverage demands significant time, making it impracticable for routine experiments.

From this point of view, it would be desirable to develop a simplified LS approach, which is based on a smaller set of wavelengths but retains much of the quality of deconvolution based on the full set of wavelengths. Not just any set of wavelengths can be used for such a simplified LS analysis, and more advanced error analysis, which takes into account the difference between the original and fitted transients at each wavelength, as well as the noise level of individual components, is needed to establish the reliability of such a set. With the right

set of wavelengths, a simplified LS approach need employ no more wavelength measurements than the DW approach, but it fully accounts for the spectral interference of individual components. Under certain circumstances, the number of wavelengths required is even less. This will be presented elsewhere.

4.4. Significance of observed quantitative differences between DW and LS analyses

Our use of LS analysis of the cytochromes of the bc_1 complex has shown that the traditional DW method does not fully compensate for overlapping absorbance bands, and errors are introduced that can be significant under circumstances in which the main absorbing component undergoes small changes and, as a result, other absorbing species contribute more significantly. LS analysis is therefore especially important under these conditions. Such situations can be expected in the presence of specific inhibitors, or at pH values outside the normal range, and at extreme redox potentials, as well as in chromatophores from mutant cells. It is also of particular importance for cyt b_L, which rarely accumulates to a significant level due to its low midpoint potential and to high throughput rates. Below, we consider the significant differences in the kinetics of individual components of wild type bc_1 complex, revealed by LS deconvolution.

In most cases, the kinetics of P870 measured at the single wavelength, 542 nm, give a small excess change, compared to that derived from LS deconvolution. However, when $P870^+$ is more fully rereduced, as seen in chromatophores from the cyt c_2 superproducer even under multiple flash activation, we observed an almost 3-fold difference in the amplitude and kinetics of P870 measured after multiple flashes (Fig. 5) due to spectral "contamination" from cytochromes.

LS deconvolution revealed that the delay before the start of b_H heme reduction is significantly shorter than that determined using the DW approach, especially at high redox potentials, when the Q pool is fully oxidized, e.g., 400 μs instead of 800 μs at E_h = 280 mV. Earlier measurements by the DW method found that the experimental variation is of similar magnitude [52], but the difference described here, between the two analysis methods, is internally consistent. The delay seen under these oxidizing conditions is due to all processes leading to the formation of ubiquinol in RCs and its delivery, by diffusion, to the Q_o site of the bc_1 complex [53]. The LS analysis therefore indicates that contributions from these processes are somewhat faster than assumed previously. At lower potentials as the Q pool is reduced and QH_2 is available immediately, the discrepancy between the two analyses becomes less, e.g., 250 μs instead of 300 μs at E_h = 150 mV.

The step-changes seen in the DW analysis of hemes b_H and b_L are contributed by both *c*-cytochromes and P870, and by residual electrochromic changes, and their amplitudes therefore depend on redox poise, relative concentrations, etc.

As described for the second flash kinetics of Fig. 3, where the *b*-heme changes are small, DW analysis "confuses" b_H with cyt c_{tot} and possibly with P870, while LS analysis determines cyt b_H accurately even in the presence of spectral interference. One can expect that similar confusion occurs under other frequently used conditions, where the concentration change of b_H is small:

(i) During the initial lag and early onset kinetics of cyt b_H reduction, the concentration changes are small, and contributions from cyt c_{tot}, P870 and residual electrochromic changes significantly modify the measured kinetics. The negative "dips" seen by the DW method immediately after the first and second flashes in Fig. 3 are due to these changes, and their minimization is the main reason why the corrected kinetics of cyt b_H have a shorter delay.

(ii) When b_H heme is reduced by multiple flashes in the presence of antimycin, the first flash induces almost complete cyt b_H reduction, and subsequent flashes cause only small changes of cyt b_H. Therefore, the relative input of other components increases, leading to distortion of the cyt b_H kinetics in the second and later flashes.

(iii) The reduction of cyt b_H via the Q_i site in the presence of inhibitors of the Q_o site is usually small at pH < 8 [54,55]. Thus, again, the relative input of other components is large. Correct assessment of the extent of b_H reduction, in this situation, is essential for determining the equilibrium constant between Q_i and the b_H heme. This is an excellent example of a situation where small changes are critical to examining the mechanisms underlying the Q-cycle.

The Q-cycle model has provided a road map for exploring the bc_1 complex mechanism but, despite the general consensus, there is still much to be desired in the way of detailed molecular understanding. In order to develop further molecular insight, the quantitative behavior of the model must be well founded. Only then can the experimental data provide a firm basis for comparison with structural and computational expectations. It is clear from the current work that adequate quantification – especially of amplitudes of changes during turnover, necessary for determining equilibrium constants – will require better spectral resolution and analysis than is provided by the difference-wavelength approach. For the purple bacteria, previous approaches to spectral separation of bc_1 components have had significant success. Even here, however, the least squares analysis presents a significant improvement over the difference wavelength method. In other systems, notably the mitochondrial bc_1 complex, the methods of spectral separation are less evolved and the LS approach should provide a substantial advantage, well worth the effort of developing the high quality of component spectra needed to apply it.

5. Conclusions

To validate the traditional difference-wavelength (DW) approach for assaying the cytochrome components of the bc_1 complex, we compared it to a least squares (LS) analysis of electron transport in *Rhodobacter sphaeroides* chromatophores, based on newly determined difference spectra of all individual components of cyclic electron transport, in situ and in isolated bc_1 complex. Multiple sets of kinetic traces, measured at

different wavelengths in the absence and presence of specific inhibitors, were analyzed by both LS and DW approaches.

Our LS analysis of the cytochromes of the bc_1 complex showed that the traditional DW method does not adequately compensate for overlapping absorbance bands. As a result, significant error can be introduced when simple spectral separation of the component of interest is attempted, especially in the case of cyt b_L, or under circumstances in which the main absorbing component undergoes small changes, and other absorbing species have more significant contributions. Of course, for changes of sufficient amplitude, the traditional DW method can provide a description of the kinetics of cyt c_{tot} and cyt b_H with an accuracy that frequently allows useful mechanistic conclusions to be drawn.

Although the current work characterizes the LS spectral analysis under uncoupled conditions and without separating the two c-type cytochromes, the method is not limited by the number of components. Extension to the separation of contributions from cyt c_1 and cyt c_2 will be described separately. The method is also applicable to coupled conditions when electrochromic changes in the membrane pigments further complicate the spectrum [56]. This is currently under development, but an initial application of this approach was presented in Shinkarev et al. [22].

The paper contains multiple examples of the utilization of the LS method that demonstrate results that advance our understanding of the bc_1 complex. These include:

- Determination of spectra of individual components in isolated bc_1 and significant corrections to spectra in chromatophores from *Rb. sphaeroides* (especially for cyts c_1 and b_L).
- The applicability and accuracy of the traditional DW approach were tested for the analysis of kinetics of individual components under several different experimental conditions in the absence and presence of different inhibitors. Many conditions were identified for which the standard DW approach is inadequate or is expected to fail.
- Kinetics and amplitudes of changes due to the b_L heme in chromatophores were better resolved by LS analysis, while simplistic application of the DW approach was shown to fail.
- Similarly, the flash-induced kinetics of the b_H heme were resolved by the LS approach, but distorted by the DW approach.

While some of these examples do not change significantly our general understanding of the bc_1 complex function, they provide the basis for significantly more challenging examination of cyt bc_1 turnover, and essential methodology for meaningful measurements of the kinetics and thermodynamics of the b_L heme in situ.

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