Ultrafast photochemistry of the bc_1 complex

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We present a full investigation of ultrafast light-induced events in the membraneous cytochrome bc_1 complex by transient absorption spectroscopy. This energy-transducing complex harbors four redox-active components per monomer: heme c_1, two 6-coordinate *b*-hemes and a [2Fe-2S] cluster. Using excitation of these components in different ratios under various excitation conditions, probing in the full visible range and under three well-defined redox conditions, we demonstrate that for all ferrous hemes of the complex photodissociation of axial ligands takes place and that they rebind in 5-7 ps, as in other 6-coordinate heme proteins, including cytoglobin, which is included as a reference in this study. By contrast, the signals are not consistent with photooxidation of the *b* hemes. This conclusion contrasts with a recent assessment based on a more limited data set. The binding kinetics of internal and external ligands are indicative of a rigid heme environment, consistent with the electron transfer function. We also report, for the first time, photoactivity of the very weakly absorbing iron-sulfur center. This yields the unexpected perspective of studying photochemistry, initiated by excitation of iron-sulfur clusters, in a range of protein complexes.

Introduction

The respiratory electron transport chain comprises a number of membrane proteins, which couple electron transfer from donors derived from nutrients to the ultimate acceptor O_2 (in aerobic respiration) to the build-up of a membrane potential used as a driving force for ATP synthesis. These protein complexes are connected by lipid-soluble or water-soluble charge carriers and contain flavin, Fe-S centers and/or heme cofactors as redox intermediates. In particular, three heme-containing protein complexes are key components of the respiratory chain: the ultimate electron acceptor cytochrome *c* oxidase (complex IV), the membrane complex from which its electrons are derived, the bc_1 complex (complex III), and a cytochrome *c* protein that shuttles between these two.

Although heme proteins are not functionally light-active, their dynamic processes have been extensively studied by flash photolysis methods. Indeed, absorption of a photon by ferrous heme can lead to high quantum yield dissociation of external ligands, such as O_2, NO and CO[1], as well as of internal heme ligands[2-4]; the latter invariably rebinding on a picosecond time scale. Furthermore, direct photooxidation of heme has been reported, but only with a very low quantum yield[5, 6].

Of the heme protein complexes in the respiratory chain, only cytochrome *c* oxidase and cytochrome *c* have been investigated in detail using time-resolved, including ultrafast, spectroscopy. These studies involved mostly dynamics of external ligands that can bind as competitive inhibitors, and also methods devised to use ligand dissociation for studying, with high time resolution, electron transfer within cytochrome *c* oxidase[7, 8] and between cytochrome c and cytochrome oxidase.[9, 10]. Until recently, such studies were not available for the isolated cytochrome bc_1 complex, which binds external ligands only to a very limited extent, and not in a functional manner.

The bc_1 complex is a homodimeric membrane protein complex that catalyzes electron transfer from the membrane-soluble two-electron donor quinol to the water-soluble one-electron acceptor cytochrome *c* whilst building up a transmembrane proton gradient via a Q cycle. Each monomer contains one [2Fe-2S] center, a *c*-type heme (heme c_1) and two 6-coordinate *b*-type hemes (hemes b_L and b_H). Electrons from quinol binding in the Q_o binding site are shuttled to cytochrome *c* via the [2Fe-2S] cluster (bound to the mobile Rieske protein) and heme c_1, and in parallel via hemes b_H and b_L to a quinone in the Q_i binding site.[11]

Recently, the first ultrafast absorption study of a bc_1 complex was reported by Chauvet and coworkers[12], who analysed transient spectra in the Q band spectral region under conditions where the c_1 heme was reduced and the *b* hemes were either oxidized or partially reduced. The authors found that in ferrous heme c_1 axial ligand dissociation and picosecond rebinding occurs as in other *c*-type heme proteins. However, interestingly it was proposed that in the ferrous *b*-type hemes no ligand photodissociation, but extremely high-quantum yield photooxidation with subsequent re-reduction occurs in ~7 ps; a property suggested to be relevant for phototherapeutic strategies.

Intrigued by this dogma-breaking property and by the potential possibility to resolve ultrafast functional electron transfer reactions in a cytochrome bc_1 complex, here we performed an extensive investigation of the photochemistry of a bacterial complex. Transient spectra of the bc_1 complex in the full visible range, obtained under different excitation conditions and in three different, well-defined, redox states were analysed. They are compared with spectra from reference proteins, in particular the 6-coordinate *b*-type heme-containing cytoglobin. We show that internal ligand dissociation and rebinding occur in the ferrous *b*-type hemes, but significant photooxidation does not occur. We moreover characterize the heme-CO geminate binding properties associated with the *b*-type hemes. Finally, we unexpectedly find evidence for a novel transient signal that can be assigned to the Rieske iron-sulfur center.

Experimental

The bc_1 complex from *Rhodobacter capsulatus* was purified as described previously[13] and suspended in 50 mM Tris buffer, pH 8.0, 150 mM NaCl, 0.01% n-Dodecyl β-D-maltoside, 20% glycerol at a protein concentration of ~20 µM (in monomers) in 1-mm optical path length cells. The fully oxidized complex was generated by adding 50 µM ferricyanide (for the experiments under 390-nm excitation these complexes were also prepared in the absence of oxygen, with very similar results). Partly and fully reduced complexes were generated by degassing the

sample in cells sealed with gastight rubber stoppers and adding sodium ascorbate (5 mM) or sodium dithionite (~1 mM), respectively. The CO-bound form was generated by exposing the fully reduced complex to 1 atm CO in the cell's head space.

Human cytoglobin was prepared as described[14] and suspended at ~30 µM in 50 mM Tris, pH 8.0, 150 mM NaCl, degassed and reduced with sodium dithionite. Reduced horse heart myoglobin was prepared as described[15] in 100 mM phosphate buffer, pH 7.4.

Steady-state spectra were obtained using a Shimadzu UV-Vis 1700 spectrophotometer. Ultrafast broad-band transient absorption experiments at a 500 Hz repetition rate, were performed as previously described[16]. Pump pulses centered at 520 or 570 nm were generated using a home-built, non-collinear optical parametric amplifier, and centered at 390 nm by frequency doubling of the fundamental beam. To ensure sharp cut-offs, the pump pulses were spectrally tailored using suitable interference filters. The sample was mounted in a sample holder thermostated at 8 °C and continuously rastered by a Lissajous scanner.

GLOBAL ANALYSIS OF THE DATA IN TERMS OF MULTIEXPONENTIAL FUNCTIONS, AND TAKING INTO ACCOUNT THE DISPERSION OF THE PROBE BEAM, WAS PERFORMED USING GLOTARAN.[17]

Results

Steady-state spectra

Figure 1A shows steady state spectra of the bc_1 complex in different oxidation states. In the initial state of the purified enzyme, the *b* hemes are fully oxidized and the c_1 heme partly reduced (not shown). From this state, addition of ferricyanide yields the fully oxidized form, which is relatively featureless in the Q band region. Addition of ascorbate to the initial state of the enzyme complex leads to full reduction of the c_1 heme[18], characterized by the typical Q band absorption maxima at 553 and 524 nm. Anaerobic addition of dithionite results in the additional reduction of the *b* hemes with Q band absorption maxima at 560 and 530 nm, typical for 6-coordinate (bis-histidine) *b*-type hemes.

Figure 1B displays the difference spectrum associated with oxidation of both *b* hemes as constructed from the steady-state spectra in Fig. 1A. This spectrum is in agreement with similarly constructed spectra in the literature[12] and spectra for the individual hemes obtained by electrochemical methods[19]. This spectrum is overlaid with a 6-coordinate minus 5-coordinate ferrous *b*-heme spectrum resulting from subtracting spectra from the fully reduced hemoglobins cytoglobin (6-coordinate bis-histidine bound[20]) and myoglobin (5-coordinate histidine bound). We note that, whereas the difference spectra closely overlap in part of the Q band region, they are dominated by strong spectral shifts in opposite directions within the Soret band.

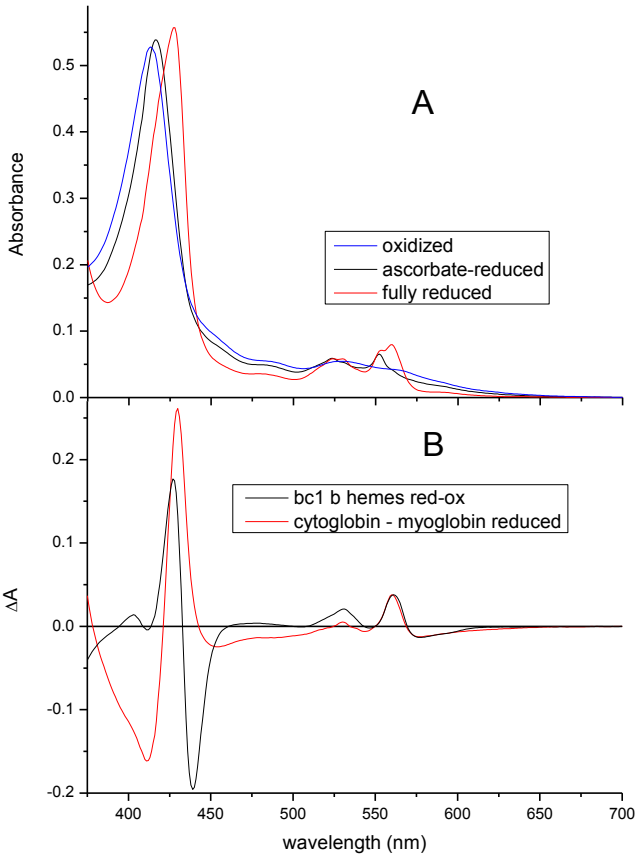
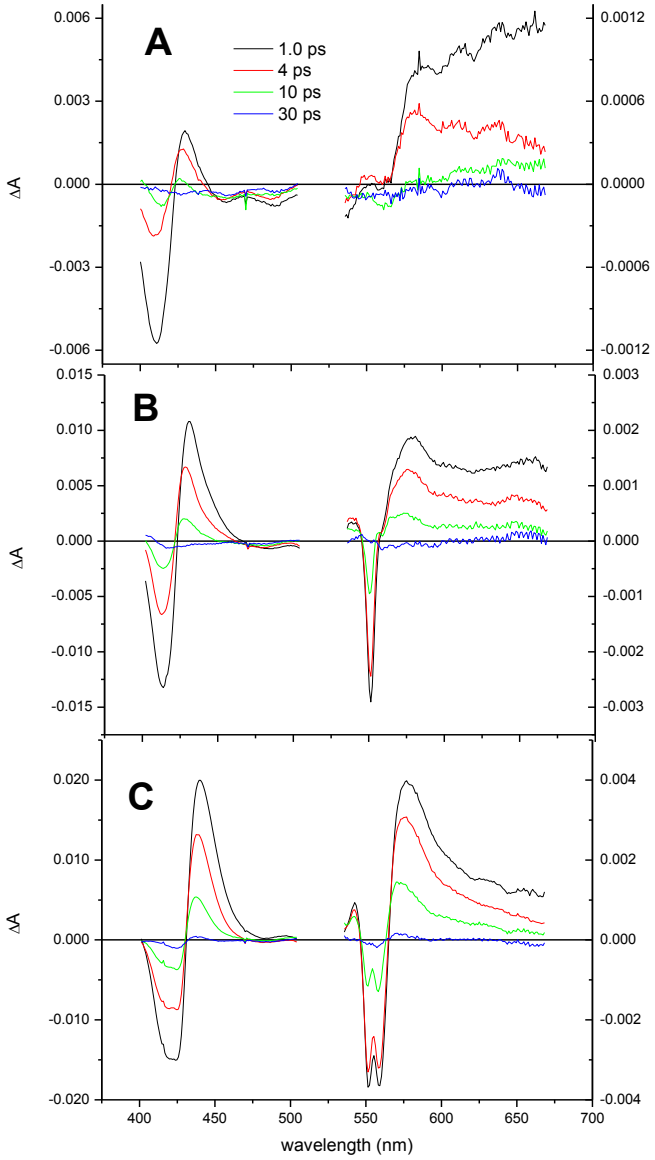


Fig. 1. (A) Absorption spectra of the fully oxidized, ascorbate reduced and fully reduced form of bc_1. (B) Difference spectra of the fully-reduced and ascorbate-reduced forms, corresponding to the reduction of the *b* hemes. Also shown is the 6-coordinate-minus 5-coordinate spectrum of *b*-hemes obtained from subtraction of the spectra of ferrous cytoglobin and ferrous myoglobin. The spectra are normalized at the 560 nm peak.

Fig. 2. Transient absorption spectra after different delay times upon excitation at 520 nm of oxidized (A), ascorbate-reduced (B) and dithionite-reduced (C) bc_1. In all panels, the spectral region above 500 nm (corresponding to the right axes) is depicted at a 5 times expanded scale. Note the different ΔA scales in the different panels.

Time-resolved spectroscopy under Q band excitation

Fig. 2 shows transient absorption spectra of the different redox forms of bc_1 obtained upon excitation at 520 nm, in the Q band region. All forms are dominated by red-shifted bands, both in the Q-band and in the Soret band region. The signals almost fully decay on the picosecond timescale. The transient spectra of the c_1-reduced form (Fig. 2B) display minima at 414 and 551 nm. For the fully reduced form (Fig. 2C) an additional sharp minimum is observed at 558 nm and the Soret minimum is broadened and red-shifted to ~425 nm. These negative absorption features roughly mirror the ground state absorption bands of the related ferrous species, in particular in the Q band, indicating that they are dominating the transient spectra. Consistently, the signals observed for the fully oxidized form are substantially weaker (Fig. 2A), but non-negligible. This implies that the signals of the ascorbate-reduced form (Fig. 2B) cannot



be solely ascribed to the ferrous c_1 heme, but also contain contributions from the ferric *b* hemes.

The full data sets were analysed in terms of decay associated spectra (DAS). For the reduced forms, a minimum of three exponential phases were required, with time constants of ~0.3 ps, ~1.3 ps and ~5 ps, These should be regarded as mean timescales on which the processes in different hemes take place. The faster phases, corresponding to blue shifting of induced absorption bands, presumably predominantly reflect photophysical relaxation processes, including vibrational cooling[3, 12, 21]. Here, we focus on the spectral characteristics of the slower processes on the time scale of a few picoseconds, leading to recovery of the initial states (Fig. 3).

Excited, fully oxidized bc_1 displays a relatively weak ~3.1 ps phase, characterized by a decaying red shift. Such phases have been observed before in both ferric 6-coordinate *b*-[5] and *c*-type[22, 23] single heme protein complexes and can be ascribed to ground state cooling. The excited c_1-reduced complex displays a much stronger signal with a time constant of ~5 ps. This signal resembles the corresponding phase observed in cyt *c* (Fig. 3) and attributed to rebinding of the dissociated distal methionine[2, 4]. It can therefore, in majority, be assigned to the same process in the ferrous c_1 heme, in agreement with a previous assessment based on the analysis of the Q band region of a partially c_1-reduced bc_1.[12]

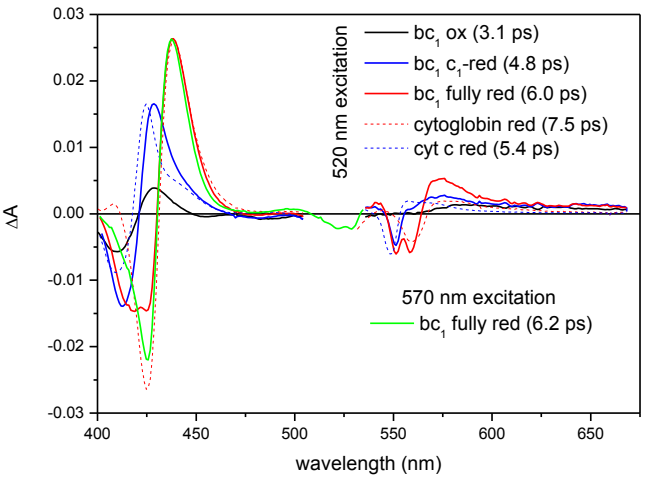


Fig. 3. Decay associated spectra of the longest picosecond phases of the bc_1 complexes at different oxidation states upon Q band excitation. The 520 nm-excitation phases correspond to the data of Fig. 2. For comparison, corresponding DAS for reduced cyt *c* and cytoglobin under identical excitation conditions, as well as for fully reduced bc_1 upon 570 nm excitation, are also shown, normalized on the Soret induced absorption bands. The corresponding decay times are indicated in brackets.

The 6 ps phase, associated with relaxation of the fully reduced enzyme, clearly contains contributions of both, the *b* hemes and the c_1 heme. The time constant should also be regarded as an averaged time associated with the different hemes, as the time constants appear very close. As the c_1-associated ligand rebinding process takes ~5 ps, the time constants of the processes associated with the *b* hemes are probably somewhat longer than 6 ps. Spectrally, the contributions of the ferrous *b* hemes are characterized by red shifts, both in the Q bands and in the Soret bands. Whereas the possible origins of these signals cannot be readily determined from the Q band region, the red-shift of the Soret band is in agreement with photodissociation and rebinding of axial *b*-heme ligands, but not with heme *b* photooxidation (Fig. 1B). Indeed, comparison of the 6-ps phase with the corresponding 7.5 ps phase in ferrous cytoglobin (containing a 6-coordinate bis-His *b* heme), indicates a similar origin of the *b* heme component of the bc_1 complex. The cytoglobin signal is thought to originate from dissociation of an axial ligand, as in neuroglobin and other 6-coordinate ferrous *b*-type heme complexes.[6] We conclude that excitation of the *b* hemes in the cytochrome bc_1 complex leads to dissociation of their axial ligands and their rebinding in ~6 ps. This conclusion is at variance with an assessment based on analysis of similar experiments on partially reduced bc_1 complexes from yeast that was limited to the Q band region.[12]

The Soret band evolution was also studied in separate experiments upon 570 nm excitation, at the red side of the Q

band region. These yielded very similar results, but for fully reduced bc_1 the *b* hemes are more selectively excited (cf. Fig. 1A). Indeed, the ~6-ps phase under these conditions is closer to the corresponding spectrum of cytoglobin (Fig. 3), further strengthening our assessment of this phase as reflecting internal ligand rebinding.

Time-resolved spectroscopy under Soret excitation

With the aim to determine the full spectral evolution in the visible region, we also performed experiments under excitation at the blue side of the Soret band. Fig. 4 shows selected transient spectra for the fully oxidized and fully reduced bc_1 complexes. The spectra after a few picoseconds display similar features as those under Q band excitation. As the oxidized *b* hemes and the reduced c_1 heme absorb quite strongly in this region, the amplitude of the signal for the fully oxidized complex is relatively high, and for the fully reduced complex the features attributed to the *c*-type heme are stronger.

Interestingly, the 2-ps spectra of Fig. 4 appear to be offset with respect to their counterparts under Q band excitation (Fig. 2). This is particularly apparent in the 450-570 nm region for the oxidized complex and between 475-500 nm for the reduced complex. At longer delay times, after decay of the features also observed under Q band excitation, a broad featureless signal, with a maximum at ~500 nm remains that decays on a much longer time scale, ~180 ps. Figure 5 shows the analysis in terms of decay associated spectra. To our knowledge, such a broad signal in this spectral region cannot be assigned to any known heme-related state. The fact that it is not observed under Q band excitation of the bc_1 complexes also strongly indicates that it is not heme-related. Moreover it is not observed in other heme proteins under 390-nm excitation (data not shown).

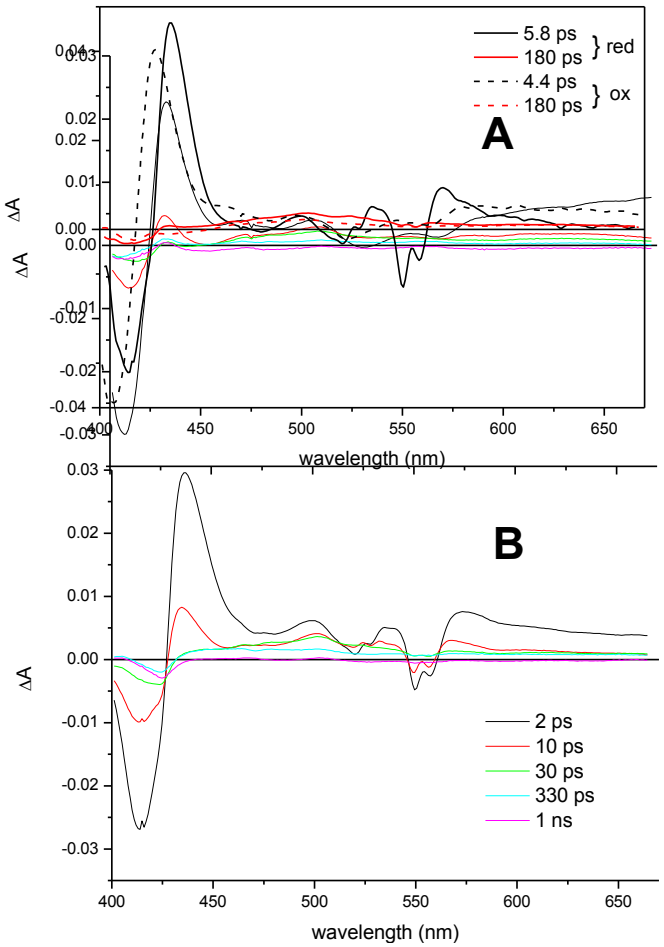
Fig. 4. Transient absorption spectra after different delay times upon excitation at 390 nm of oxidized (A) and dithionite-reduced (B) bc_1.

Fig. 5. Decay associated spectra of the ~5 ps and 180-ps phases of oxidized and fully reduced bc_1 complexes under 390 nm excitation (data from Fig. 4).

We therefore propose that the signal originates from another light-absorbing component of the bc_1 complex. Indeed, beside the three hemes, the complex harbors a [2Fe-2S] cluster. Both oxidized and reduced [2Fe-2S] centers absorb at 390 nm (extinction ~5000-10000 $M^{-1}cm^{-1}$)[24-27]. Hence we provisionally assign this signal to an Fe-S cluster photoproduct. We note that the shape of the observed signal approximately corresponds to that expected for the difference between oxidized and reduced [2Fe-2S] clusters[24-27]. One possibility is therefore that the signal arises from photo-oxidation and subsequent re-reduction of reduced [2Fe-2S] clusters. The fact that nevertheless a similarly shaped, yet significantly weaker, signal is observed in the oxidized complex (Fig. 5) may be due to incomplete oxidation of the Fe-S clusters under our conditions.

CO dissociation and rebinding

It is known that CO can bind to the *b*-type hemes in the purified bc_1 complex.[28, 29] Fig. 6 shows the spectral changes associated with exposure of the fully reduced bc_1 complex to CO The decrease in amplitude of the 560-nm peak indicates that CO can bind, to a limited extent that is quantified below, to the *b*-type hemes, as reported previously.[28, 29] We note that comparison of the difference spectra with those of the prototypical 6-coordinate and 5-coordinate proteins cytoglobin and myoglobin shows that CO replaces an internal distal



histidine residue (like in cytoglobin[20, 30]), rather than binding to a fraction of 5-coordinate hemes, as suggested previously.[28]

The Soret-band transient spectra observed after excitation of the bc_1-CO complex and the corresponding data analysis are shown in Fig. 7. The spectra strongly decay on the timescale of a few picoseconds and the dominant 6.3-ps phase can be ascribed to rebinding of the photodissociated internal ligands of the *b* and c_1 hemes, as in the fully reduced enzyme. Note that the relative amplitude of the blue shoulder in the bleaching of this component, assigned to the c_1 heme, is higher than in the fully reduced enzyme under identical excitation conditions (Fig. 3, green curve), in agreement with the notion that less bis-histidine *b* heme is present in this form. Comparison of the amplitude of the corresponding components at 421 nm (the isosbestic point for the corresponding component in the c_1-reduced enzyme (Fig. 3)) indicates that the amount of bis-histidine *b* heme is reduced by ~25% upon exposure to CO.

Additional phases with very similar spectral properties, and time constants of 130 ps (40%) and > 1 ns (60%, the actual rate and extent of this decay phase could not be determined due to the limited time domain), were retrieved. These have the typical characteristics of 5-coordinate minus CO-bound *b*-hemes and can be safely assigned to rebinding of dissociated CO. We conclude that a substantial fraction of the dissociated CO rebinds, in a multiphasic way, within a few nanoseconds. This finding indicates that on the picosecond and early nanosecond timescale the dissociated CO remains close to the heme in a configuration allowing rebinding.

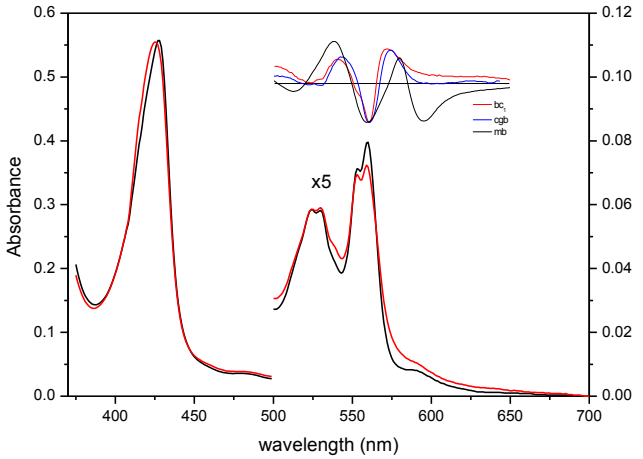


Fig. 6. Absorption spectrum of the fully reduced (black) and CO-reduced (red) forms of bc_1. The spectral region above 500 nm (corresponding to the right axes) is depicted at a 5 times expanded scale. *Inset:* CO-minus-reduced difference spectrum overlaid with corresponding spectra of cytoglobin and myoglobin, normalized at 560 nm.

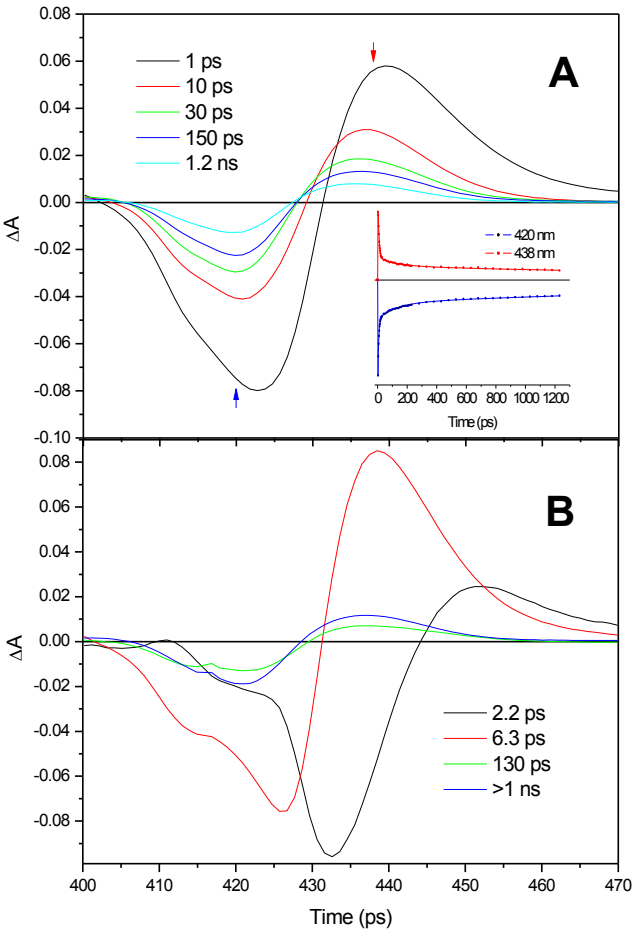


Fig. 7. Transient absorption spectra after different delay times (A) and Decay Associated spectra (B) upon excitation at 570 nm of the bc_1-CO complex. The *Inset* in panel A shows the kinetics at selected wavelengths.

Discussion

It is well established that excitation of 6-coordinate ferrous (Fe^{2+}) hemes leads to dissociation of a distal ligand. This has been demonstrated by transient vibrational spectroscopy for cytochrome *c*[2, 4], and inferred by transient absorption spectroscopy in the Soret band for a variety of *b*-type heme containing proteins[6]. In all studied proteins the internal ligand, which is part of the protein backbone, rebinds to the heme predominantly on the timescale of ~5-7 ps, with additional phases on the tens of picoseconds timescale in some cases[6]. The present work demonstrates that the hemes of the bc_1 complex are no exception in this respect. Our data on the *R. capsulatus* complex are in full agreement with those reported previously for the yeast complex[12], but extended to a larger spectral range, a variety of excitation conditions and three well-defined redox conditions. In agreement with the earlier assessment[12], we found that in the c_1 heme an axial ligand (presumably methionine) dissociates and rebinds in 5.4 ps. The extension of the spectral domain to the Soret region, where signals arising from ligand dissociation and heme oxidation can be uniquely distinguished (Fig. 1B), allowed us to asses that heme-histidine dissociation occurs in the *b* hemes as well, and that reformation of these bonds occurs in

~6-7 ps. The latter conclusion contrasts with that of reference 12, in which the analysis was limited to the Q-band region. In the latter work, the analysis was based on subtraction of data obtained under two redox conditions in which the hemes were differently partly reduced, and it was implicitly assumed that ferric hemes do not contribute to the signal. This assumption is not confirmed by our experiments (Figs. 2A, 3). We note that similar complications may have played a role in the recent proposal of photooxidation and picosecond re-reduction of heme *f* in the b_6f complex (which plays an analogue role to the bc_1 complex in the plant photosynthetic electron transfer chain), which was based on similar subtraction procedures of Q band spectra.[31]

Our results demonstrate that the photoresponse of the *b*-type hemes in the bc_1 complex is very similar to that of bis-histidine *b*-type hemes in mono-heme proteins, such as cytoglobin in this work (Fig. 3), neuroglobin and cytochrome b_{559}[6]. The universal ~5-7 ps heme-histidine rebinding times presumably reflect the intrinsic barrierless binding process.[6] In neuroglobin, a second, ~threefold slower rebinding phase was also observed, that was suggested to be associated with distal heme pocket flexibility, required for the functional accommodation of external ligands.[6] The absence of such a phase in the bc_1 complex, as well as in the photosynthetic membrane protein cytochrome b_{559}[6], is consistent with the pure electron transfer function of these hemes, which requires a rather rigid heme environment. We note that in cytoglobin monophasic ligand rebinding kinetics were observed under our experimental conditions. We foresee to investigate whether this property is influenced by varying the reduction state of intramolecular disulphide bonds, which is known to modulate ligand affinity[32].

The finding that in the CO-binding fraction of the *b* hemes recombination of dissociated CO with heme occurs predominantly within 1 ns is also consistent with a rigid heme pocket, as expected for electron transfer proteins. By comparison, CO rebinding on this timescale also occurs in minimally modified CO-binding variants of the electron transfer protein cytochrome *c*[10].

This work was inspired by suggestions of photo-initiated heme redox changes in the bc_1 complex. Whereas these were not observed, unexpectedly, weak but relatively long-lived signals assignable to changes in the electronic state of the [2Fe-2S] center were seen under blue excitation. To the best of our knowledge, light-induced changes in Fe-S centers are unprecedented. They may provide a novel means to study electron transfer processes in Fe-S cluster-containing proteins. The ground state absorption of these centers is broad and has an extinction coefficient over an order of magnitude lower than these of individual hemes and is therefore essentially masked in the bc_1 complex. The strong heme absorption both a) limits the sample concentration that can be employed to study these low-extinction features and b) masks events occurring up to the tens of picoseconds timescale. In future studies, we will therefore investigate Fe-S proteins that do not contain other colored cofactors.

Conclusions

We have shown that in all ferrous hemes of the bc_1 complex photodissociation of axial ligands takes place and their rebinding occurs in 5-7 ps. The binding kinetic of internal and external ligands are indicative of a rigid heme environment, consistent with the electron transfer function of the hemes. Furthermore, our work yields a novel unexpected possibility of studying photochemistry initiated by excitation of iron-sulfur clusters.

Acknowledgment

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