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Review

Time-resolved infrared spectroscopic studies of ligand dynamics in the active site from cytochrome *c* oxidase☆

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The catalytic site of heme–copper oxidases encompasses two close-lying ligand binding sites: the heme, where oxygen is bound and reduced and the Cu_B atom, which acts as ligand entry and release port. Diatomic gaseous ligands with a dipole moment, such as the signaling molecules carbon monoxide (CO) and nitric oxide (NO), carry clear infrared spectroscopic signatures in the different states that allow characterization of the dynamics of ligand transfer within, into and out of the active site using time-resolved infrared spectroscopy. We review the nature and diversity of these processes that have in particular been characterized with CO as ligand and which take place on time scales ranging from femtoseconds to milliseconds. These studies have advanced our understanding of the functional ligand pathways and reactivity in enzymes and more globally represent intriguing model systems for mechanisms of ligand motion in a confined protein environment. This article is part of a Special Issue entitled: Vibrational spectroscopies and bioenergetic systems.

1. Introduction

Cytochrome *c* oxidase (CcO) *aa*$_3$ is the terminal oxidase of cellular respiration in higher eukaryotes and is equally present in a large number of microorganisms. This enzyme complex catalyzes the four-electron reduction of molecular oxygen to water and during this process, the redox energy is stored as a transmembrane proton gradient that is subsequently used to generate adenosine triphosphate (ATP) [1,2]. On the catalytic subunit I, CcO *aa*$_3$ contains a six-coordinated low spin heme *a* that mediates electron transfer from exogenous cytochrome *c* toward the active site of the enzyme and a bimetallic center composed of the five-coordinated heme *a*$_3$ and the adjacent Cu_B ion, which is located 5 Å away (Fig. 1). Located on a different protein subunit is the redox-active copper center Cu_A that is formed by two copper atoms. Upon reduction, Cu_A holds an electron by sharing it between both copper atoms.

While cytochrome *c* oxidase *aa*$_3$ is the best known representative of the superfamily of heme–copper oxygen reductases, several different bacteria-specific representatives exist, like the *caa*$_3$ and *ba*$_3$ oxidases, probably best characterized from the bacterium *Thermus thermophilus* [3,4] or the cytochrome *cbb*$_3$ oxidases with three extra subunits besides the catalytic one; two of them containing additional redox centers, a diheme and a monoheme cytochrome [5]. Whereas this review primarily concerns ligand dynamics in CcO *aa*$_3$, these bacterial oxidases provide interesting systems for comparison. In CcO *aa*$_3$, heme *a*$_3$ is the binding site for molecular oxygen and its reaction intermediates during its reduction and also binds competitor ligands like CO and NO, which act as biological messengers. Because heme binds CO (and NO) tightly, the accessibility of these ligands to heme *a*$_3$ must be well controlled, with a possible role of Cu_B in regulating ligand traffic. Before binding to heme *a*$_3$, O_2 or other small-molecule ligands bind to Cu_B intermittently [6]. The reverse reaction, involving ligand dissociation from heme *a*$_3$ and eventual transfer out of the protein via prior binding to Cu_B, can be studied using flash photolysis, exploiting the photolability of the heme–ligand bond. As CO forms a stable complex with cytochrome *c* oxidase, it has been widely used to investigate the mechanisms, dynamics and the pathways open to oxygen and other exogenous ligands to and from the active site.

Time-resolved resonance Raman spectroscopy has been used to investigate the dynamics after CO dissociation from reduced CcO on picosecond and longer time scales, while UV–Vis absorption experiments have been performed on femtoseconds to milliseconds time scales [7,8]. These experiments probe only the heme sites directly, while the reduced Cu_B site is generally spectroscopically silent. Electronic absorption and resonance Raman studies on cytochrome *c* oxidase *aa*$_3$ are further complicated by contributions from both cytochromes *a* and *a*$_3$, which can be difficult to distinguish.

In contrast, infrared spectroscopy is uniquely suited as a probe for the reactions of CO with CcO, particularly the ligand binding reactions of Cu_B. The high oscillator strength of the CO stretch infrared absorption band around 2000 cm^{-1} combined with the high sensitivity of its frequency and bandwidth to changes in binding and protein environment,

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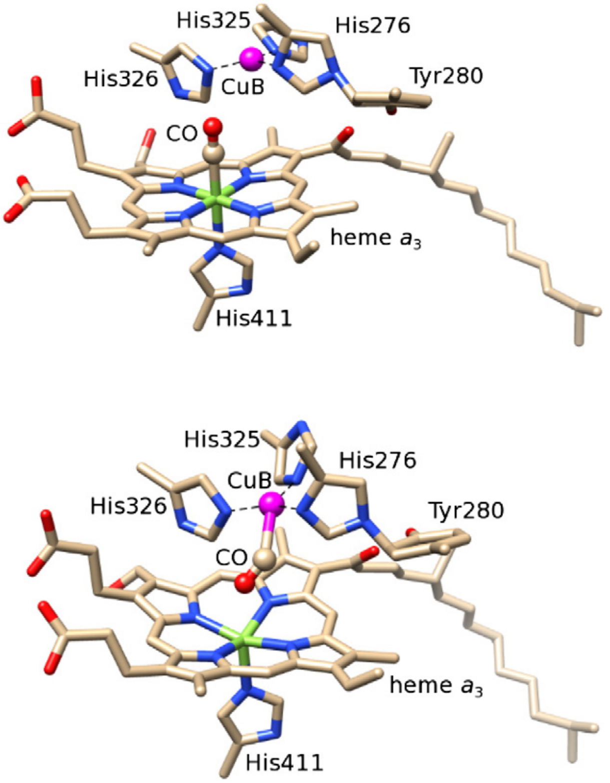

Fig. 1. Structures of the active site of CcO aa_3 from *Paracoccus denitrificans*, with CO liganded to heme a_3 (top) and Cu_B (bottom). The structures are snapshots taken from molecular dynamics simulations described in Ref. [18]. The dashed lines represent hydrogen bonds.

as well as the fact that this band stands background-free, well separated from the other vibrational transitions of the protein complex, make it an ideal probe of ligation reactions.

The structural dynamics of biological reactions in protein complexes span many orders of magnitude in time. Although the *overall* rate of O_2 turnover by cytochrome *c* oxidase *aa*$_3$ is quite moderate (~600 s^{-1}), the process encompasses ultrafast events of ligation of small molecules to the binuclear site. Different from standard descriptions of enzymatic catalysis, where the transition state between reactant and product is reached by thermal, stochastic motions, in the ultrafast time domain, the protein moiety and cofactor motions leading to altered conformations can be coherent rather than stochastic in nature. Such coherent motions may play a key role in controlling the accessibility of the transition state and explain the high efficiency of the reaction. Cytochrome *c* oxidase, with its two ligand binding sites distant of only a few Ångstrom, thus provides a unique environment in which the mechanisms of ligand transfer and bond formation can be studied directly.

2. Technical aspects

Most of the phenomena described in this review have been observed using time-resolved mid-infrared techniques. The common steady-state technique in this spectral range is Fourier Transform Infra Red (FTIR). Here, a mostly continuous, broad-band light source is used and spectral resolution is obtained using a Michelson interferometer. In this way, no losses due to dispersive or spectral selection optics occur and the entire available infrared light intensity from the, essentially black body, continuous light-source can be exploited for detection. Time resolution while maintaining spectral resolution can be obtained by coupling this technique with a pulsed visible light source (typically a nanosecond laser) that triggers a perturbation of the system. For heme proteins, and in particular here heme–copper oxidases, this optical perturbation can result in the dissociation (photolysis) of a ligand bound to the heme in the active site, in particular for CO or NO as ligands. Recently, electrochemical pulses have also been explored in combination with surface-enhanced IR spectroscopy to study the protein response to electron transfer events [9].

For processes slower than the scan time, full interferograms at different times after the perturbation can be measured (rapid scan); for higher time-resolution the inverse approach is taken and kinetics at different interferogram positions are taken, allowing full recovery between scan steps (step scan) [10,11]. The latter method has a time resolution in principle in the nanosecond range, limited by the detector response and the laser pulse length. It is available on several commercial instruments and has been used for a number of studies on ligand dynamics near the active site of CcO.

If spectral resolution is not required, intense continuous monochromatic infrared sources can be used for probing. For instance, Dyer and coworkers used a tunable IR diode laser in early experiments on the microsecond kinetics of the Cu_B-bound CO complex in CcO [12].

For subnanosecond resolution, ultrashort visible excitation and infrared detection pulses are used and time resolution is achieved by mechanical scanning of the delay between these pulses (pump-probe principle). Here, with femtosecond pulses, intense infrared pulses with spectral widths up to hundreds of cm^{-1} and temporal widths in the range of 10–200 fs can be generated using non-linear optical techniques like difference frequency generation [13] or optical rectification [14]. Spectral resolution of the probe pulse can be obtained by dispersion in an infrared polychromator and detection with (typically 64-pixel) infrared detector arrays [15]. Alternatively, interferometry can also be used for spectral resolution [16]; note that in this case the interferometric scan range (10 ps full range for a 3 cm^{-1} spectral resolution) can contribute to the effective temporal resolution. A promising new method for spectrally resolving short infrared pulses is its upconversion to the visible domain by mixing with a chirped near-infrared pulse, and subsequently detecting it after dispersion using a charge coupled device (CCD) [17]. The higher pixel density and dynamic range of these less costly detection devices allow high spectral resolution and sensitivity. This method has been used to temporally resolve the femtosecond transfer dynamics of CO between the two binding locations in the CcO active site [18].

We note that the high yields of infrared light generation by downconverting ultrashort visible light can also been exploited for experiments with low time resolution, avoiding the need for interferometric spectral characterization. This approach has been applied in recent work on the protein response to CO dissociation from Cu_B in bovine heart CcO, where the delay between a nanosecond visible pulse and a broad-band femtosecond infrared probe pulse was electronically controlled, and the probe beam, highly attenuated by a strongly absorbing background, was spectrally resolved via dispersion and detector arrays [19].

Exploiting visible transient absorption spectroscopy, for example in colored heme proteins, low frequency molecular vibrations can in principle also be observed in real time as coherent modulations of the spectra [20,21]. This method has been used to demonstrate ballistic population transfer during CO migration within the CcO binuclear active site [22].

3. Ultrafast ligand dynamics in the binuclear site of cytochrome *c* oxidases

As stated before, photodissociation studies of CO have played a major role in elucidating the ligand binding reactions of the binuclear center in cytochrome *c* oxidases. Early flow-flash experiments based on CO photodissociation from CcO *aa*$_3$, pioneered by Gibson and Greenwood [23], established the first kinetic evidence for short-lived binding intermediates [24].

FTIR experiments demonstrated that, below 180 K, photodissociated CO migrates to and binds to Cu$_B$ in mitochondrial *aa*$_3$ preparations [25,26]. Einarsdottir et al. [27] also observed in fully reduced CO-cytochrome oxidase *ba*$_3$ from the bacterium *T. thermophilus* that upon photolysis CO binds quantitatively to Cu$_B$ (in its reduced form, Cu$_B^+$) at temperatures between 20 and 300 K. Nanosecond time-resolved IR experiments at ambient temperature with eukaryotic cytochrome *c* oxidase *aa*$_3$, where CO is bound to the heme *a*$_3$ iron, demonstrated that, upon photodissociation, CO binds quantitatively within 100 ns to Cu$_B$ [12]. Additional experiments implicated a Cu$_B$–CO intermediate in the *reverse* reaction as well, suggesting an implication of Cu$_B$ in ligand transfer [28]. A parallel kinetics study showed that, in addition to its established redox function, Cu$_B$ acts as a gateway to heme *a*$_3$ and that coordination to Cu$_B$ is an obligatory mechanistic step for CO (and presumably other small molecule ligands) that enter the *a*$_3$ heme site and leave the protein after photodissociation [7,28].

Einarsdottir et al. [7] studied the picoseconds to hours kinetics following CO photolysis of the flash-induced photodissociation and rebinding of carbon monoxide in CcO *aa*$_3$–CO by time-resolved infrared (TRIR), transient ultraviolet–visible (UV–Vis) spectroscopy at room temperature and by Fourier transform infrared (FTIR) spectroscopy at low temperature. These studies confirmed binding of photodissociated CO to Cu$_B$ at room temperature by the TRIR absorption at 2061 cm^{-1} due to the C – O stretching mode of the Cu$_B$–CO complex (see below). The data support a pre-equilibrium between free CO in solution and a Cu$_B$–CO transient intermediate prior to the formation of the cytochrome *a*$_3$–CO complex. This pre-equilibrium also occurs in the reaction with O$_2$, prior to O$_2$ binding to the heme [12,28–30], validating the functional importance of CO studies.

Induced infrared linear dichroism (polarization photoselection) measurements show the angle between the heme normal and the C – O bond vector to be 20° for Fe$_{a3}$–CO and 55° for Cu$_B$–CO [31], in excellent agreement with structural modeling [22]. Dyer et al. [22], in a TRIR experiment with picosecond temporal resolution, investigated the rate of photo-initiated transfer of CO from heme *a*$_3$ to Cu$_B$ by monitoring the development of the transient Cu$_B$–CO absorption. They found, that in less than 1 ps a stationary Cu$_B$–CO spectrum develops, which is constant until CO dissociates from Cu$_B$ on a microsecond time scale (see below). These observations indicate that complete transfer of photodissociated CO with full formation of the Cu$_B$–CO complex in the ground state occurs within 1 ps. The CO transfer is much faster than would be expected for CO diffusion toward Cu$_B$. This suggested that CO remains in the heme *a*$_3$–Cu$_B$ site and is transferred from heme *a*$_3$ to the Cu$_B$ center in a concerted manner with the motion of the heme pocket. Concomitant with CO movement in the active site, heme *a*$_3$ evolves from a low-spin state toward a five-coordinate high-spin state (in less than 5 ps; [32]), presumably involving doming of the heme.

Taking advantage of the fact that CO-bound cytochrome *c* oxidase can be formed in steady state, and that ligand transfer can be initiated and synchronized by photon absorption, Liebl et al. [22] studied the heme dynamics during the photo-initiated ultrafast transfer of carbon monoxide from heme *a*$_3$ to Cu$_B$ in bacterial cytochrome *c* oxidase *aa*$_3$ using visible multicolor femtosecond spectroscopy. This study showed that the ground state of the unliganded heme *a*$_3$ species is populated in a stepwise manner in time, indicating that the reaction is mainly governed by coherent vibrations of ~47 cm^{-1}. Supported by molecular dynamics simulations, the authors provided evidence that heme *a*$_3$ and Cu$_B$ move in a concerted way during the transfer of CO to Cu$_B$. It has been argued before that this process is ballistic, taking a minimal time of 300 fs [31] (corresponding well with the half-period of the ~47 cm^{-1} reaction-coordinate motion) and allows for optimizing the probability of CO for colliding with Cu$_B$, a doorstep on the way into or out of the active site. Beyond the interest for CcO itself, these results provided the first evidence for the driving of a reaction in a protein complex by coherent motions and suggest the functional importance of vibrational coherence operating on a femtosecond/picosecond timescale.

These findings were further corroborated and extended by a subsequent femtosecond time-resolved and fully spectrally resolved visible pump-mid-infrared probe experiment. Treuffet et al. [18] were able to directly time-resolve the CO ligand transfer process in the bimetallic active site from the heme *a*$_3$-iron to the neighboring Cu$_B$ atom using the full vibrational signature of Fe – CO bond breaking and Cu$_B$ – CO bond formation. Differential transmission spectra showed an onset of the appearance of the CuB-bound species after 200 fs, followed by a 450-fs exponential rise (Fig. 2). Trajectories calculated by molecular-dynamics simulations display a similar behavior. Both experimental and calculated data equally suggest a ballistic contribution to the transfer process.

Other than CO, nitric oxide (NO) also acts as a competitive ligand for O$_2$ at the binuclear active site, although the dynamics of NO in this site are less well understood. Ultrafast transient absorption spectroscopy of NO from heme *a*$_3$ in bacterial CcO *aa*$_3$ showed that the formation of the unliganded heme *a*$_3$ ground-state occurs in stepwise manner, similar to what was previously observed for CO and interpreted in terms of ballistic ligand motions in the active site [22] and that two NO molecules can bind to the binuclear site at micromolar concentrations [33]. This contrasts with mitochondrial *aa*$_3$, where *in the physiological range* only one NO molecule binds to heme *a*$_3$ and no transient binding to Cu$_B$ occurs. Only at very high (millimolar), non-physiological concentrations a second NO is accommodated in the active site of mitochondrial CcO *aa*$_3$ [34]. These differences may relate to differences in the physiological relevance of the CcO–NO interactions in both oxidases and await further clarification.

4. CO transfer out of the CcO *aa*$_3$ active site

Because of the substantial dissociation enthalpy of the Cu$_B$ – CO bond (~30–40 kJ/mol, [26,35]), at cryogenic temperatures a virtually stable Cu$_B$–CO complex can be formed by illumination of the CcO–CO complex, where CO is initially bound to heme *a*$_3$. Cryogenic steady-state light-

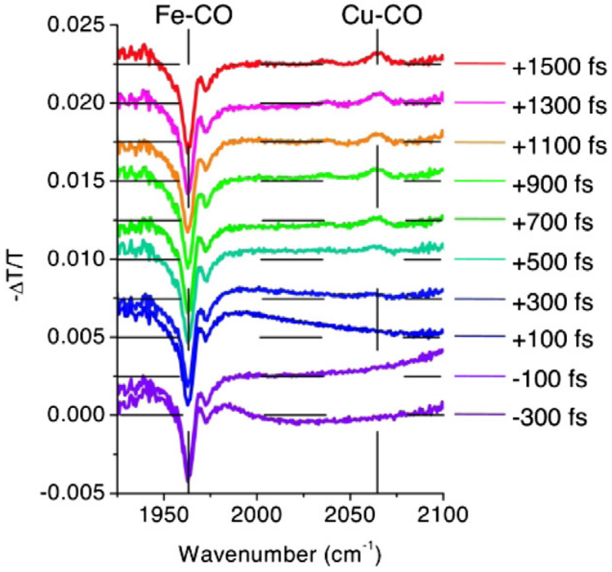

Fig. 2. Differential absorption spectra on the femtosecond timescale upon CO dissociation from heme *a*$_3$ in mammalian CcO *aa*$_3$. The spectra are displaced vertically for clarity (horizontal dashed lines indicate the origin). Vertical dashed lines show the position of the Fe–CO and the Cu$_B$–CO vibrational peaks. The oscillating features and bleach signals at negative delay times, that extend prior to the pump–probe overlap time, are due to perturbed free induction decay effects.
Reproduced from Ref. [18]; see also for further details.

minus dark FTIR spectroscopy had allowed determination of the Cu_B-bound CO stretching frequency at ~2062 cm^{-1} [25,26]. The first observation of the Cu_B–CO complex at room temperature was achieved by probing the transient kinetics at this frequency upon photodissociation of the a_3 – CO bond in mammalian CcO [12]. The transient Cu_B–CO complex was found to have a lifetime of ~2 μs; this time corresponds to escape from the active site and is limited by the thermal dissociation rate of the Cu_B – CO bond. Later, it was found that this process is also associated with a small shift in the visible spectrum of the hemes, presumably mostly heme a_3 [7,36], in principle facilitating kinetic studies of the process.

The response of the protein upon CO dissociation from heme a_3 was initially studied with frozen samples [37,38], which slows down the reactions and facilitates time-resolved FTIR approaches. Rapid-scan FTIR experiments at 268 K that could not resolve the Cu_B–CO intermediate, indicated substantial changes in the amide I bands and furthermore, along with a mutagenesis approach, a change in the environment of a glutamate (E-I286) in the vicinity of the active site that is part of the D proton channel [38]. Subsequent step-scan experiments at 253 K, where CO migration from Cu_B out of the protein occurs only partially and is slowed down to ~80 μs, showed that environmental changes of the above-mentioned glutamate also occur upon release of CO from Cu_B [37]. Yet, as such changes do not occur in an equivalent bacterial enzyme [37], they may not be functionally relevant.

At room temperature, the response of the protein to CO binding to and subsequent release from Cu_B has been monitored only very recently by Yoshikawa and coworkers using dedicated non-FTIR instrumentation, in mammalian CcO *aa*$_3$ [19]. The authors assigned the most prominent signals in the amide I region (unassigned and also quite different in the previous low-temperature work [37]) to structural changes of a serine residue interacting with the hemes and suggested such changes to play a role in proton traffic upon transient binding of the O_2 substrate to Cu_B. Interestingly, although the CO absorption region does not indicate transient accommodation of CO along the escape pathway on a timescale longer than the ~2 μs Cu_B–CO dissociation time (in agreement with Cu_B to heme a_3 transfer being rate-limiting for stable a_3–CO complex formation [6]), slower microsecond phases were mentioned for the protein response [19]. Yet uninterpreted, they presumably reflect slow protein rearrangements upon CO release from the active site.

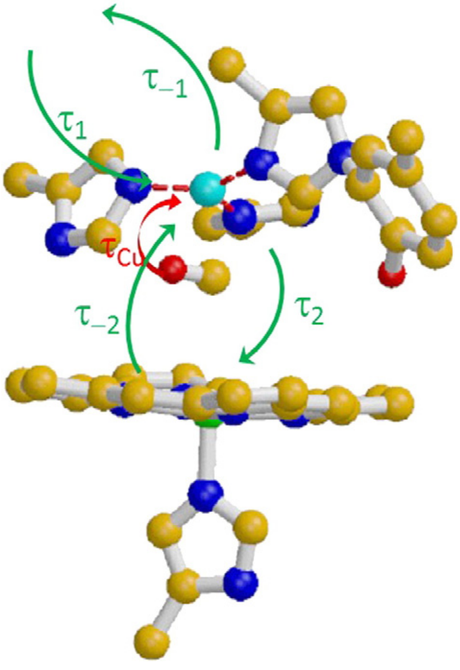

Fig. 3. Scheme for CO transfer reactions in heme–copper oxidases. The green arrows correspond to the thermally activated reactions. The red arrow corresponds to CO binding to Cu_B from the photodissociated form.

5. CO migration in other oxidases

As outlined above, in CcO *aa*$_3$ enzymes the Cu_B–CO temporal intermediate can be readily detected by ultrafast spectroscopic techniques, but it has proven difficult to study this intermediate at near-physiological temperatures using conventional step-scan FTIR due to its short lifetime. In principle, characterization of this intermediate state is more favorable in the *ba*$_3$ oxidase from *T. thermophilus*, where the Cu_B moiety has a very high affinity for CO. Indeed, in the equilibrium *ba*$_3$–CO complex in ~30% of the enzyme complex, CO is liganded to Cu_B rather than to heme a_3 [39,40], an unusual property possibly related to the more compact heme a_3–Cu_B active site structure [41]. A further favorable property for studying the Cu_B–CO state in this enzyme is the relatively strong CO-stretch absorption properties in the Cu_B-bound form (extinction ~60% of that of the heme a_3-bound form vs ~15% in CcO *aa*$_3$ [27]). The Cu_B-bound CO manifests itself as a single IR transition at 2053 cm^{-1} [40], at significantly lower frequency than the main (transient) Cu_B – CO band in the *aa*$_3$ enzyme at ~2062 cm^{-1}. This difference may be related to the difference in affinity. Upon photodissociation of CO from heme a_3 it binds to Cu_B [40], presumably on the timescale of a few hundred femtoseconds as in the *aa*$_3$ enzyme, although this has not been directly measured. The subsequent off-equilibrium residence time of CO on Cu_B is ~30 ms, ~four orders of magnitude longer than in the *aa*$_3$ enzyme. Varotsis and coworkers observed that the recovery of the heme a_3 – CO bond occurs quasi-simultaneously, indicating virtually no escape of CO from the active site [40,42,43], in contrast to the *aa*$_3$ enzyme. The same authors have also interpreted this observation in terms of a more complex model, in which the coincidence of the rates is fortuitous, CO does fully escape to the solvent and the photochemically generated Cu_B–CO has properties distinct from the steady-state thermal equilibrium Cu_B–CO population [40]. However, the induced transient CO-stretch band is indistinguishable from the equilibrium steady-state band [40] and the rebinding should have the properties of a bimolecular process, which has not yet been investigated. Altogether, at present the intrinsic rate of CO escape from Cu_B to the solvent in the *ba*$_3$ oxidase remains unknown, but must be lower than 30 s^{-1}. The large difference to the *aa*$_3$ enzyme, where this rate is ~3.10^5 s^{-1}, can be due to both, the intrinsic Cu_B – CO bond strength and the more restrained binuclear site [41], which presumably hinders escape. The ensemble of available data from IR and visible spectroscopies can be described in terms of a minimal model as presented in Fig. 3 and Table 1.

A small fraction of the photodissociated CO appears not to bind to Cu_B, but is docked in a non-bonding state in the protein [42,44]. By similarity to the IR spectral properties, this state was suggested to resemble the (fully populated) initial docking state in myoglobin, where CO can be oriented in two directions near-parallel to the heme-plane [45]. Ultrafast IR experiments will be required to investigate these similarities. On the microsecond time scale, this docked CO relaxes in a complex manner, suggested to involve propionate isomers on the basis of molecular dynamics simulations [46], to a spectrally silent form (such as CO in solution). Recombination of this relaxed form appears to occur on a similar (~30 ms) time scale as the transient Cu_B–CO form [44]; therefore the pathway of transfer from Cu_B to heme a_3 may actually pass via this state. The mechanism how the population of this state would efficiently compete with binding to Cu_B remains to be understood.

The CO transfer from heme a_3 to Cu_B is accompanied by spectral changes assigned to heme propionates [40] and tyrosine residues in their vicinity [47]; the latter were suggested to be those involved in catalytic proton translocation. Finally, in the presence of highly substoichiometric amounts of O_2 in the dithionite-reduced *ba*$_3$–CO complex, slow changes (hours timescale) in the IR properties of the

Table 1
Room temperature time constants of CO transfer reactions in various oxidases in the framework of the minimal reaction scheme of Fig. 3, at 1 mM CO in solution.

	τ_{Cu}	τ_1	τ_{-1}	τ_2	τ_{-2}
aa_3	~500 fs[a]	17 μs[b]	2 μs[b]	1 ms[b]	40 s[b]
ba_3	nd	~10 ms[f]	>30 ms[c] ~0.4 s[e]	30 ms[c]	~60 ms[d]
cbb_3	nd		>1 ms[g]	130 μs[g]	
caa_3	nd	60 μs[h]	120 μs[h]	30 ms[i]	

[a] From Ref. [18]. τ_{Cu} does not correspond to a classical rate, but to a ballistic reaction time.

[b] From Ref. [36].

[c] From Ref. [40].

[d] Assuming equilibration between CO binding on heme a_3 and Cu_B is at the origin of the ~30% steady-state Cu_B–CO concentration.

[e] From overall off rate of 0.8 s^{-1} [61] and τ_{-2}/τ_2 equilibrium.

[f] From overall off rate of 0.8 s^{-1} and overall equilibrium binding constant of $1.1 \cdot 10^5$ M^{-1} [61].

[g] From Ref. [52].

[h] From equilibration time of ~40 μs and ~1/3 net CO dissociation from Cu_B [53].

[i] From Ref. [53].

steady-state Cu_B–CO population were observed, but no significant changes in the rate of transfer from Cu_B to heme a_3 were detected [48]. These observations were hypothesized to reflect the properties of Cu_B as an O_2 entrance channel.

Interesting further comparative cases are provided by the cbb_3 enzyme from *Pseudomonas stutzeri* and the caa_3 oxidase from *T. thermophilus*. These bacterial enzymes dispose of a different electron transfer chain used for reducing the active site, but the general properties of the heme–Cu_B active site are similar to other heme–copper oxidases. As observed for cytochrome *c* oxidase ba_3, these enzymes display NO reductase activity. The cbb_3 enzyme has a very high O_2-affinity and is thought to support respiration in microaerophilic environments. Although the interaction of NO with these enzymes has not yet been studied by IR spectroscopy, the dynamics of CO are of considerable interest.

In the cbb_3 enzyme of *P. stutzeri*, the structure of the heme b_3–Cu_B active site [49] and the IR spectral properties of the carbonmonoxy-form [50] appear similar as in other oxidases. As in ba_3 oxidase, a relatively high extinction of the Cu_B–CO form is observed, but there is no evidence for a steady-state Cu_B–CO population. Remarkably, although CO is also known to bind to the *c*-type cytochrome in *P. stutzeri* [5,51], no IR signal associated with this cytochrome *c*–CO species was reported. Upon light-induced transfer to Cu_B, CO rebinds directly to heme b_3 predominantly in ~130 μs [52] and no CO appears to escape the active site within ~1 ms. These properties resemble those of the ba_3 enzyme; the ~1 order of magnitude faster rebinding presumably reflects a weaker Cu_B – CO bond. We also note that analysis of CO rebinding experiments of *P. stutzeri* cbb_3 oxidase in the visible range yields a rate-limiting transfer time of CO from Cu_B to heme b_3, which is ~1 order of magnitude longer than the 130 μs obtained by transient IR spectroscopy [51]. As the latter corresponds to a (small) fraction that has escaped the active site, this may be due to protein heterogeneity.

In the caa_3 oxidase, in contrast to the ba_3 enzyme, no steady-state Cu_B–CO population is observed. Interestingly, Varotsis and coworkers observed only partial (~one third) CO escape from transiently populated Cu_B–CO that occurs in ~40 μs (an order of magnitude slower than in aa_3), followed by full rebinding of CO to heme a_3 in ~30 ms [53]. In the simplest view, this can be seen as a transient pseudo-equilibrium between CO in solution and Cu_B–CO (Table 1, Fig. 3), in rough agreement with early work mentioned by Woodruff in a review [54], although more complex reaction schemes are also possible [53]. Thus, the CO transfer properties of caa_3 oxidases are between the full escape found for aa_3 and no or little escape in ba_3/cbb_3 enzymes.

6. NO migration in ba_3 oxidase

Generally, all three oxidases ba_3, cbb_3 and caa_3 display substantially higher ligand retention properties at the Cu_B site than the aa_3 enzyme (Table 1). This characteristic may correlate with the NO-reductase activity of these three bacterial enzymes, which requires colocalization of two small (NO) ligands in the binuclear active site. Unfortunately, studying the NO migration in oxidases by transient IR spectroscopy is very challenging and has so far only been attempted for the ba_3 enzyme complex. The ba_3 oxidase displays considerable NO reductase activity and therefore the dynamic interaction of the reduced enzyme with NO is of high interest. In particular, the co-presence of two close-binding sites for NO may favor the reaction ($2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$). By transient absorption spectroscopy in the visible range, it has been shown that NO dissociated from heme a_3 rebinds in ~15 ns, presumably after the transient formation of a Cu_B–NO complex, but that 20% of the NO rebinds in ~50 μs, either from an intra-protein docking site or from the solvent [55]. Thus interestingly, the ba_3 enzyme represents a rare case in heme proteins where the probability of escape from the heme pocket is larger for NO than for CO, presumably because the Cu_B – NO bond is more thermolabile than the Cu_B – CO bond [56]. Unfortunately, in step-scan FTIR experiments of the ba_3–NO complex with a 5 μs time resolution, the dissociated fraction could not be detected [57]. Indeed, whereas NO is also infrared-active, the transition of heme-liganded NO has a 2–3 fold lower extinction than the corresponding CO transition and moreover falls in the amide I transition region of the protein moiety [58]. To investigate directly whether NO transiently binds to Cu_B at all and identify the properties of any other intra-protein docking site, experiments with higher time resolution and sensitivity are required.

7. Conclusions and perspectives

The binuclear active site of cytochrome *c* oxidase complexes provides a unique environment in which the dynamics of small gaseous molecules can be investigated and time-resolved infrared techniques are highly suited to study these processes directly. Here we give an overview, both of the ballistic regime of ligand transfer from the heme (where oxygen and competitor ligands CO and NO bind) to the Cu_B site. We moreover address the extreme versatility of ligand dynamics from the Cu_B site on. These studies are of high interest, both for the understanding of the functional ligand pathways and reactivity in the enzymes, and more broadly as a biophysical chemistry model system for mechanisms of ligand motion in a confined protein environment. In view of the outlined, very favorable, infrared spectroscopic properties of CO, the grand majority of this work was performed with this physiological, non-reactive, signaling molecule as a ligand. Studies implicating the NO signaling molecule, which can also act as a substrate in various bacterial oxidases, are far more challenging, both for spectroscopic reasons and due to the stability of the complexes, but should become feasible with oxidases. This is especially the case in the ultrafast time domain, where relatively high signals and rich reaction dynamics are expected and where pioneering studies have already been performed in other heme proteins [59,60].

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