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Review

Ultrafast dynamics of ligands within heme proteins

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Abstract

Physiological bond formation and bond breaking events between proteins and ligands and their immediate consequences are difficult to synchronize and study in general. However, diatomic ligands can be photodissociated from heme, and thus in heme proteins ligand release and rebinding dynamics and trajectories have been studied on timescales of the internal vibrations of the protein that drive many biochemical reactions, and longer. The rapidly expanding number of characterized heme proteins involved in a large variety of functions allows comparative dynamics–structure–function studies. In this review, an overview is given of recent progress in this field, and in particular on initial sensing processes in signaling proteins, and on ligand and electron transfer dynamics in oxidases and cytochromes.

Keywords: Heme proteins; Femtosecond spectroscopy; Molecular dynamics; Heme-based sensors; Oxidases; Cytochrome

1. Introduction

Heme proteins are involved in a large diversity of biological functions, including transfer and storage of molecular oxygen, catalysis, electron transfer and signaling. The heme groups are bound to the protein matrix, and mostly well shielded from the solvent. The heme is associated with the protein via axial bonding between the heme iron and one or two residues; one being usually (but not exclusively) a histidine (Fig. 1). The sixth position can be occupied by external ligands, for ferrous heme in particular O_2, NO and CO. Functional processes in many heme proteins involve ligand binding and release or exchange of external or internal ligands. Physiological dissociation of the Fe–ligand bond occurs by thermal activation; propagation of functional changes may occur subsequently in competition with rebinding of the ligand to the heme. The probability of the latter event to occur depends on the ligand and in particular is determined by the structure and dynamics of the protein matrix. The present review deals with the dynamics of heme, ligand and protein, as well as their interplay, on the timescale of femtoseconds (1 fs = 10^{-15} s) and picoseconds. This timescale is typically relevant for conformational changes occurring in the near vicinity of the heme.

Thermal breaking of heme-ligand bonds, or arrival of external ligands in the protein matrix, cannot be synchronized on this timescale. Whereas in principle single-molecule approaches associated with ultrafast time resolution and long observation times (bond breaking events typically take place with rates on the millisecond to second timescale) could be used to study dynamics, experimental methods in practice invariably rely on the fact that heme iron–ligand bonds can often be dissociated, with high quantum yield, using photons in resonance with the electronic transitions of the heme. Photodissociation is not a physiological event, but can be used to mimic functional thermal dissociation and dissociation of ligands that are not physiological (like CO in myoglobin), the dynamics of which are used as a probe for the heme environment. Thus optical pump-probe techniques are employed, in which a short visible photodissociation pulse (typically ~50 fs) is combined with a second non-perturbing electromagnetic pulse which probes properties like absorption (visible or infrared), Raman scattering, or X-ray diffraction, as a function of time delay between the pulses. Femtosecond absorption spectroscopy is now a mature technique that is routinely used in many laboratories.

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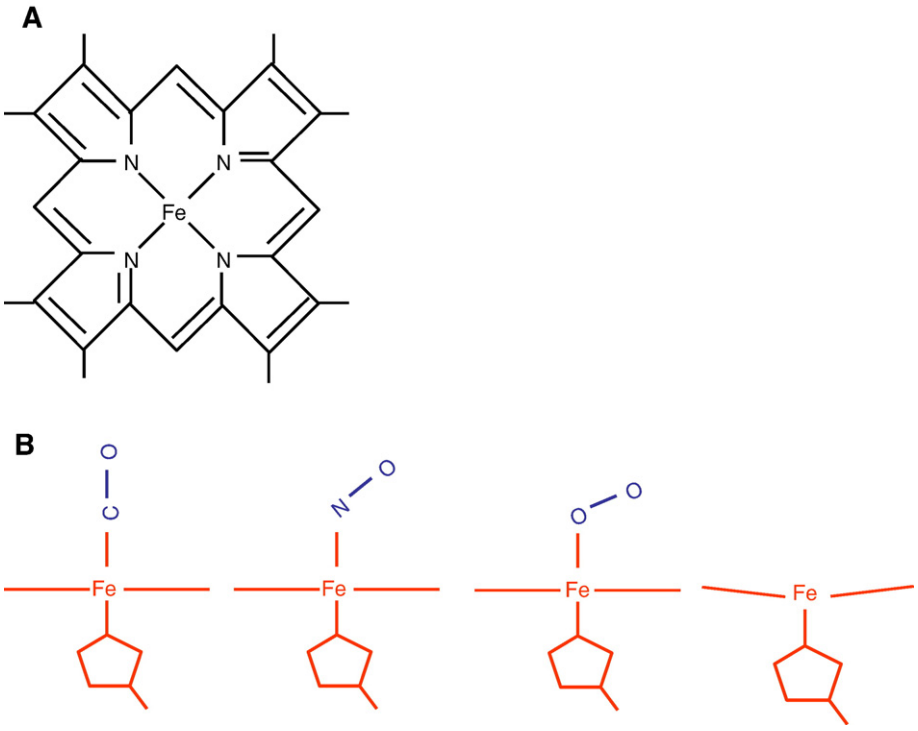

Fig. 1. General structure of heme. (A) View on the heme plane. Lateral chains (not drawn) are associated according to heme type. (B) Schematic view in the heme plane, of heme liganded with histidine and with CO, NO, and O_2 or no ligand in the 6th position. In isolated complexes, the Fe–X–O angles are ~180°, 145° and 120°, respectively; within a protein they may deviate due to environmental constraints. The 5-c heme plane is slightly "domed" (Fe ~0.5 Å out of plane).

Advanced ultrafast techniques, like those employing mid-infrared pulses, are rapidly being developed and often applied immediately to heme proteins, which form convenient model systems for studying fast protein dynamics.

Femtosecond and picosecond protein motions are also on the timescale accessible for classical molecular dynamics simulations [1]. Therefore the experimentally observed processes on macroscopic samples can often be confronted with, and the interpretation guided by, all-atom (single molecule) simulations.

The initial pioneering studies on fast processes in heme proteins were performed essentially on the mammalian 'model' oxygen storage and transport proteins myoglobin (Mb) and hemoglobin (Hb), and have been reviewed before [2–5]; a few structural and dynamic properties of Mb are summarized in Section 2. Here we focus on the rapidly expanding recent studies on a variety of heme proteins, with an emphasis on proteins involved in bioenergetic processes and signaling. In many cases, myoglobin is used for comparison. The remainder of this review is organized as follows. First, photophysical considerations involving the interaction of short light pulses with hemes anchored in proteins will be briefly reviewed (in Section 2), with mainly Mb as an example. Subsequently, an overview will be given of ultrafast studies on heme-bearing signaling proteins (Section 3), oxidases (Section 4) and cytochromes (Section 5), and a short outlook will be given.

2. Photophysical aspects

2.1. Photodissociation yield and geminate recombination

Fig. 1 shows the general heme structure. Optical excitation of 6-coordinate (6-c) hemes, via the transitions lying in the plane of the heme, leads to population of intrinsically short-lived (hundreds of femtoseconds to a few picoseconds [6,7]) excited states. These have anti-bonding character [8] and can lead to dissociation of the Fe–ligand bond within tens of femtoseconds [7]. Dissociation then leads to heme "doming" (see Fig. 1) within 1 ps [9–11].

In Mb, the quantum yield of photodissociation (number of ligands dissociated per photon absorbed) has been determined at close to 1 for CO [12,13], and ~0.5 and 0.3 for NO and O_2 respectively [14]. The latter two values were measured on the early picosecond timescale taking MbCO as a standard. For O_2, rebinding phases on the femtosecond timescale may also play a role (see Section 3.5). These values are higher than the yields of ligand ejection into the solvent, as especially for NO and O_2, the geminate recombination (recombination of the very same pairs that were dissociated, prior to the ligand leaving the protein) phases can be substantial. With intense pulses that are long with respect to the geminate recombination time (for instance nanosecond pulses for most NO-binding proteins) the yield, per pulse, of ligand escape from the protein can be increased by multiple absorption/dissociation events. However, this is

not possible for femtosecond visible pulses, where the amount of dissociated ligands cannot exceed 50%, because of repopulation of the ground state by the excitation pulse, and where saturation effects already play a role at lower intensities.

2.2. Ligand migration properties in myoglobin

Myoglobin is a ubiquitous (yet not essential for all species [15]) mammalian oxygen storage heme protein. It was the first protein with a structure determined by X-ray crystallography [16] and serves as a model system for heme-ligand interactions. The most extensively studied form is the complex poised with CO, which is more stable than the protein complexed with the physiological ligand O_2, and has a large photodissociation quantum yield (see above) and little geminate recombination. In Mb and the allosteric protein Hb (comprised of four Mb-like subunits), CO geminate recombination contributes only for a few percent to the total recombination at room temperature and occurs on the microsecond timescale [12], and a few tens of percents on the tens of nanoseconds timescale [17], respectively. The temperature dependence of this slow geminate recombination has been extensively studied by Frauenfelder's group and others to determine barrier crossings associated with conformational changes of the protein-dissociated CO system [18–20]. In several of the more recently investigated proteins, substantial picosecond and early nanosecond CO recombination phases are observed at room temperature.

In Mb, heme-bound CO is oriented near-perpendicular to the heme plane [21,22] (Fig. 2), as first assessed by femtosecond infrared polarization studies [23]. Dissociation of CO leads, within a few hundred femtoseconds, to two similarly populated docking sites close to the heme, in both of which the orientation of CO is roughly parallel to the heme plane, and that have been assigned to rotamers [24]. Assignments of the infrared spectra of the two sites have been made by combining site-directed mutagenesis and infrared steady-state cryotrapping experiments [25]. A recent elegant time-resolved two-dimensional infrared study connects two conformations of CO-bound Mb each with the two distinct configurations of the picosecond-dissociated states [26].

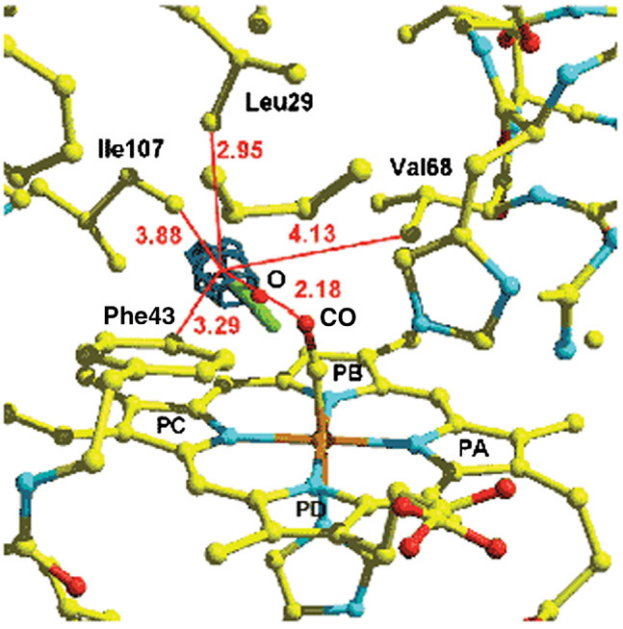

Fig. 2. Initial docking site of dissociated CO from Mb after 1 ns (blue) on the template of the CO bound structure 1BZR [21]. The green bar depicts the dissociated CO from cryophototrapping experiments at 40K. For further details see Ref. [28]. Reproduced with permission from Ref. [28]. Copyright (2001) American Chemical Society.

MbCO is the first system studied using time-resolved X-ray crystallography [27,28]. The single docking site proposed on the time scale of 1 ns in those studies, ~2Å from the bound position (Fig. 2), is consistent with the two configurations observed spectroscopically being rotamers [24]. Numerous structural rearrangement associated with CO dissociation, including heme doming have also been described. In WT Mb, CO occupies the initial docking for ~200 ns [29]. Although other factors may also play a role, rotational sequestering of dissociated CO perpendicular to its bound position may be invoked to explain the lack of geminate rebinding during this time. Subsequently CO occupies the so-called Xe(1) site located on the proximal site of the heme [28] prior to leaving the protein on the microsecond time scale. The occupation time of the initial docking site can be strongly reduced to ~140 ps by replacing Leu29 (Fig. 2) by the bulkier residue Phe [29], without geminate recombination occurring. In this case, structural studies show that the dissociated CO displaces close by residues including Phe29, thus facilitating access to cavities located further away on the distal heme side, prior to occupying the Xe(1) site as in WT. It was suggested that the intermediate cavities are part of the migration pathway in WT Mb as well, but cannot be observed in time resolved studies because of the long dwell time in the initial docking site [29]. The 150-ps time resolution in these structural studies was limited by the duration of the synchrotron X-ray pulses. Much higher time resolution can in principle be obtained by using devices employing X-ray pulses generated by femtosecond laser pulses; such 'table-top' devices are under development [30,31].

NO is more reactive towards heme than CO, and, generally, dissociated NO has a high chance to rebind to the heme before it can leave the protein. The kinetics of picosecond NO rebinding with many heme proteins are non-monoexponential and exquisitely sensitive to the heme environment. They have been extensively used to probe the involvement of specific residues in the dynamic properties of the distal environment of the heme in Mb [32–38], for a review see [4]. Initial data on native MbNO were discussed in terms of discrete as well as continuous, or gradually decreasing, distributions of rates [39], but later work showed that multi-modal distributions of rates describe the kinetics best [34], implying NO recombination to the heme from a limited number of distinct conformational states. Indeed, recently three distinct conformations of dissociated NO have been observed in a detailed time-resolved infrared spectroscopic study by Kim and Lim [40] that also allowed modelling their interconversion on the picosecond timescale. Whereas two of the three states (presumably roto-isomers) were found to be populated rapidly after dissociation, direct rebinding to the heme only

occurs via one state, which may be associated with the barrierless rebinding site (see Section 3.3). A complication arises from the finding of these authors that the intrinsic rebinding rate from this site in Mb is itself time-dependent (5–90 ps), a feature ascribed to protein relaxation. From the fact that ~7-ps monoexponential overall NO rebinding is observed in a number of proteins (Table 1), including the relatively rigid [41,42] protein cytochrome *c*, it appears that this is not a general feature for heme proteins. Further general features of heme-NO rebinding will be discussed in Section 3.3.

In the steady-state nitrosylated heme, the bond is formed between the heme iron and the nitrogen atom (Fe–N–O). Recent theoretical studies have suggested that transiently the inverse configuration (Fe–O–N) can also be formed [43]. This is an interesting possibility in view of the idea that different rotamers of dissociated NO can be populated in the same site near the heme in Mb [40]. However, experimentally such a configuration has not (yet) been identified in time resolved studies.

Only very few ultrafast studies have been performed on the $Mb–O_2$ complex. These will be discussed in Section 3.5.

2.3. Thermal effects

The photon energy deposited in the heme is only partially used to break the Fe–ligand bond. For instance estimates for the enthalpy of the Fe–CO bond in Mb range from ~22 [44] to ~35 [45,46] kcal/mol; a photon absorbed by the lowest-lying heme α bands corresponds to ~50 kcal/mol, and for the Soret band this value is ~70 kcal/mol. Thus, inevitably internal conversion processes lead to excitation of higher vibrational levels ("heating") of the heme and subsequent cooling. Whereas this effect can be minimized by excitation in the α bands, and experiments at different excitation wavelengths do not indicate strong effects in transient absorption experiments beyond a few picoseconds [14], thermal dissociation can in principle be mimicked better and without excess energy by depositing energy directly in the (infrared) transition of the Fe–ligand bond. Unfortunately the Fe–CO stretch (~500 cm^{-1}) absorbs only weakly and in particular cannot be excited exclusively. However the CO stretch of bound CO (~1950 cm^{-1}) has a strong absorption and is background-free, and energy dissipation is likely to occur mainly via population of bottleneck Fe–C vibrational levels (see below). As the binding energy of the Fe–C bond corresponds to 4–6 infrared 1950 cm^{-1} photons, multiphoton excitation of this band may be explored. Indeed vibrational ladder climbing of the CO stretch up to the 6th vibrational level has been shown possible using tailored pulses [47], though yet without succeeding in sizeable dissociation; further progress may be expected with the development of high-energy infrared sources.

The possibility to locally deposit energy in the heme by a light pulse has been used to explore energy flow within the cofactor-protein system by various vibrational spectroscopic techniques. Initially, increase in population of higher vibrational levels can correspond to local 'temperature' rises of well above 100 K, if one assumes Boltzmann equilibrium [48]. It should be kept in mind however that thermal equilibrium does not strictly exist, and re-equilibration occurs rapidly (see below); therefore the concept of temperature is inadequate in this situation. Cooling of the heme has been studied by time resolved resonance Raman (TR^3)

Table 1
Kinetics of geminate NO rebinding to ferrous native heme proteins at room temperature up to early nanosecond timescale

Protein	τ_i in ps (A_i)	Comments	Reference
Myoglobin (horse heart)	5 (0.53) 50 (0.22) 500 (0.21)	From data up to 4 ns.	[81], see also refs. [4,33,34,39,94,95,97,170–173]
Hemoglobin (human)	10 (0.77) 67 (0.23)		[39], see also ref. [174]
NO synthase (human endothelial)	36 (0.23) 400 (0.14)		[81]
Guanylate cyclase (bovine)	7.5 (0.97)	NO receptor; forms 5-c heme-NO complex	[82]
FixL heme domain (*Bradyrhizobium japonicum)*	5.3 (0.2) 20 (0.24) 220 (0.08)	Oxygen sensor	[117]
Dos heme domain (*Escherichia coli*)	5 (0.85) 20 (0.15)	Oxygen and/or redox sensor	[107]
Cytochrome *c* (horse heart)	8 (1.0)		[90], see also ref. [91]
Cytochrome *c′* (*Alcaligenes xylosoxidans)*	7 (1.0)	Forms 5-c heme-NO complex	[89]
Cytochrome *c* oxidase aa_3 (*Paracoccus denitrificans*)	200 (0.9)	Less recombination at very low NO concentrations	[76,147]
Cytochrome oxidase ba_3 (*Thermus thermophilus*)	>4000	Oxidase with NO reductase activity	[147]
Dehaloperoxidase	9 (0.56) 53 (0.44)	Globin enzyme	[36]
Neuroglobin (human)	5 (0.62) 21 (0.28) 260 (0.06)		L. Kiger & M.H.V., unpublished results

Results of fits to multiexponential decay kinetics (of the form $\sum A_i \exp(-t/\tau_i)+A_\infty$) are listed.

spectroscopy using Stokes- and anti-Stokes bands [48–53] and by near infrared absorption spectroscopy [54], and typically takes place in a multiphasic manner on the timescale of up to ~10 ps, with a strong component of 1–2 ps. Recent experiments [55,56] and simulations [57,58] with modified hemes in Mb indicate that a substantial fraction of the cooling 'pathway' passes via the propionate side chains of the heme, that are in hydrogen bonding interaction with the protein matrix. Using infrared absorption spectroscopy, the complementary heating process of the protein environment of the heme has been observed to take place in Mb and Hb in ~8 ps, and energy flow into the aqueous solvent on the tens of picoseconds range [59].

Upon dissociation of a ligand, a fraction of the excess vibrational energy is also stored in the dissociated ligand. For instance in MbCO, a few percent of the dissociated CO (characterized by the CO stretch around 2080 cm^{-1}) were found to be in the first vibrationally excited state [60]. As there are no bonding interactions with the environment, the dissipation of this excess energy occurs on a much longer timescale (several hundreds of picoseconds [61]). Thus, ligand migration in many of the processes covered by this review takes place without effective thermal exchange between ligand and protein.

Finally, when CO is bound to the heme, excess vibrational energy deposited in the CO stretch (by excitation with infrared pulses) decays in ~25 ps [47,62–64]. This value is between those for cooling of directly excited heme and for unbound heme in a protein matrix, and is presumably limited by energy dissipation via the Fe–C bond.

After dissociation of CO from Mb, relaxation processes on the time scale of ~50 ps have also been observed using Soret circular dichroism spectroscopy [65] and transient Raman spectroscopy in the ultraviolet [66]. These have been assigned to a more specific response of the protein environment to the initial conformational change of the heme.

2.4. Vibrational coherence effects

Another feature specific for the femtosecond regime is the possibility to study coherence effects [5]. The use of pulses shorter than the period of characteristic vibrations of the heme-protein system allows synchronization of vibrational motions in a macroscopic sample. In particular, dissociation of a ligand from a 6-c heme changes the equilibrium position for the Fe–His mode (~220 cm^{-1}, period ~150 fs) and the heme "doming" (Fig. 1) mode (iron out-of-plane motion, ~50 cm^{-1}, period ~700 fs), so that high-amplitude motions along those modes are initiated. Because these low-frequency vibrations are coupled to the Soret transition (the nuclear position along those modes influences the position of the optical transition), these vibrations, that can remain in phase up to several picoseconds at room temperature, can be followed in real time spectroscopically [67–71]. Upon excitation in the Soret band, vibrations on the bound ground state are also activated by the Raman effect. This can be used as a way to perform Raman spectroscopy [72], but also complicate analysis of the functional motions of the dissociated species. It has also been shown to be possible to follow the ensemble of coherent motions in three dimensions by time resolving infrared emission from heme protein crystals after visible excitation [73]. In this technique, the crystal acts as a radiating antenna and all vibrations involving charge movement (up to ~2000 cm^{-1}), not only those optically coupled to the heme, can be detected.

If the coherent motions are involved in a reaction, it is possible that the *population* transfer rate also vibrates in time [74], yielding non-exponential, stepwise reaction dynamics. This has been shown to be the case for the heme-doming mode during ligand transfer reactions in cytochrome *c* oxidase [75,76] (see Section 4.1).

3. Signaling proteins

Heme-based sensor proteins detect changes in the level of diatomic gaseous ligands. In these proteins, binding of the messenger molecules (essentially NO, CO or O_2) to heme initiates chemistry that eventually results in the organism's response to changes in ligand availability. Within the protein, ligand binding to, or dissociation from, the heme leads to modulation of the activity of an associated enzymatic domain. Thus, dissociation of the ligand in principle can trigger the signaling process and may permit characterization of short-lived intermediates in the intra-protein signaling pathway.

Heme-bearing enzymes are also involved in the production of signaling molecules. CO is a degradation product of heme, produced by heme oxygenases [77]. The important signaling molecule NO is produced *in situ* by a heme protein, NO synthase [78,79]. In the following, we will discuss advances on NO-producing and sensing enzymes in the general framework of the exquisitely sensitive NO dynamics, followed by CO and O_2 sensors.

3.1. NO synthase

The enzyme NO synthase is present in different isoforms in mammals [78,79], and produces NO and L-citrulline using O_2 and L-arginine as substrates. The active site contains a heme, which is linked to the protein via a proximal cysteine. O_2 binds to the ferrous heme, and the L-arginine binding site is nearby. The product NO can also bind to the heme, both in its ferrous and ferric state. Thus, in equilibrium with the local NO concentration, NO inhibits further NO production, a property that constitutes a regulatory mechanism [80].

Upon dissociation of NO from endothelial NO synthase (eNOS), a fraction of heme-NO pairs rebinds on the picosecond-nanosecond timescale [81]. This is the case in virtually all studied heme proteins, and reflects the intrinsic high affinity of NO for heme. However, NO escapes from the heme environment with a substantially higher yield (>50% unbound heme after 4 ns for the ferrous protein, Table 1) than in other studied heme proteins (with the exception of oxidases under some circumstances where a nearby second binding site is available, see Section 4.1). This property can be related to the NO production function of the enzyme, which requires NO to be released efficiently into the solvent. Indeed, Fig. 3 illustrates that the kinetics are in stark contrast to the very fast and efficient kinetics of NO rebinding in the NO receptor guanylate cyclase [82],

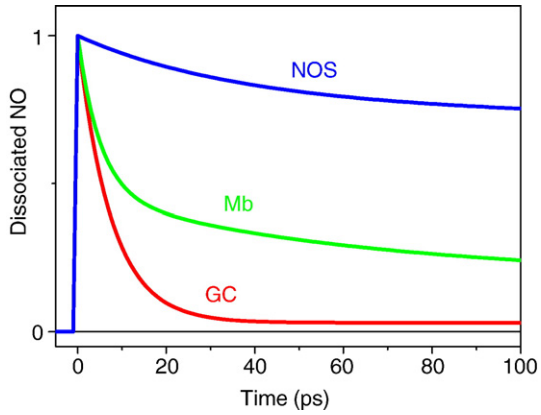

Fig. 3. Relation between NO rebinding kinetics to ferrous heme and function. NO rebinding to the NO-releasing enzyme NO synthase (blue) is slow and with low amplitude, and NO rebinding to the NO receptor soluble guanylate cyclase is fast, single exponential, and with almost unity amplitude (red). NO rebinding to myoglobin (green) is intermediate and multiexponential. The kinetic traces are based on data from Refs. [81] and [82].

which exerts the complementary function in NO signaling (see Section 3.2).

As for many other heme proteins, the geminate recombination fraction of dissociated NO displays multiphasic kinetics. This has been interpreted in terms of sequential population of sites that have progressively higher barriers for NO rebinding [81]. As has been extensively demonstrated in Mb using site-directed mutant analysis (see Section 3.3), the details of the rebinding kinetics are a very sensitive probe of the heme environment. For instance, the overall nanosecond rebinding of NO to ferric eNOS becomes dramatically more efficient in the presence of the substrate L-arginine, thus providing an additional form of regulation of the enzyme activity [81]. Along the same lines, the NO concentration itself was found to influence strongly the NO rebinding kinetics to the ferrous enzyme in the presence of the cofactor tetrahydrobiopterin (BH_4). These findings led to the proposal of an intra-protein, non-heme NO docking site, which can also be involved in the fine tuning of eNOS activity [83]. NO recombination has also been used to probe the heme environment and the BH_4 binding site of the inducible NO synthase isoform [84], and NO synthase from bacterial pathogens [85].

3.2. Guanylate cyclase

Soluble guanylate cyclase (sGC) is an NO receptor with a yet unresolved structure that is crucially involved in NO signaling in a variety of mammalian tissues [86]. Its enzymatic domain can catalyze the formation of the second messenger cyclic guanosine monophosphate from guanosine triphosphate; a reaction that is activated by binding of NO to the associated heme domain. The deoxy-form of GC contains a histidine-coordinated 5-c heme. CO can bind on the distal side to form a 6-c heme, and dissociated CO does not recombine up to several nanoseconds or more [82]; both these properties are like MbCO. Consistent with its role as NO receptor the protein does not bind O_2 [87], and has a high affinity for NO. Unlike in MbNO, NO binding to the heme is associated with dissociation of the bond between the heme and the proximal histidine, and leads to a 5-c complex. It is unknown whether NO replaces the proximal histidine or binds at the distal side of the heme. The displacement of the proximal histidine is thought to be part of the signaling pathway that leads to activation of the enzyme. Dissociation of NO from this complex leads to very rapid (7.5 ps) and efficient (virtually unity yield) recombination (Fig. 3, Table 1) [82]. As discussed above, this property contrasts with that of the NO-releasing protein NOS and can be associated, in general terms, with the receptor function of sGC. Indeed, later studies of heme-based CO and O_2 sensors (see below) also indicate highly efficient rebinding for the respective physiological ligand.

NO dissociation from GC thus leads to a 4-c heme species, an assessment confirmed by transient spectroscopic studies of engineered Mb [88]. Such a form has not been characterized in steady-state. It has thus been possible to determine optical absorption and (at least for the somewhat related protein cytochrome *c′*, see below [89]) resonance Raman properties of this species. The transient spectra also indicate that at all times before NO rebinding (to form the 5-c form), the proximal histidine remains detached from the heme [82], and therefore de-activation is not immediately initiated upon NO dissociation. Thus, the fast NO recombination aids in maintaining sGC in the activated form, and de-activation is probably induced by other factors than (thermal) NO dissociation.

The relevant fast and efficient rebinding of NO to the 4-c heme may be due to the intrinsic affinity of the 4-c heme and/or to a protein environment that does not permit escape. Comparison with similar experiments on an engineered Mb, where the proximal histidine is replaced by glycine and a 5-c NO complex can also be formed, and where multiphasic NO rebinding qualitatively similar to native Mb occurs [88], implies that the transient 4-c heme is at least not solely responsible for the fast rebinding, and that the protein environment plays an important role. Inversely, single-exponential rebinding of NO to a 5-c heme (with proximal histidine) has now also been observed in cytochrome *c* [90,91] (see Section 5).

NO binding to bacterial cytochrome *c′* leads to a 5-c heme-NO complex [92], as in sGC. The function of this protein is not yet established, but its structure is known [93]: in this protein NO replaces the proximal histidine, and thus binds at the proximal side. It has been suggested that, although cytochrome *c′* and sGC show no homology, a similar mechanism might hold for sGC [93]. NO rebinding to cytochrome *c′* was found to be similarly rapid and efficient as in sGC [89] (Table 1). Also, notwithstanding the identification of two distinct NO-binding configurations in the crystal structure [93], a single exponential rebinding phase was found. This finding is consistent with this phase corresponding to the intrinsic bond-formation time (see below). Molecular dynamics simulations are consistent with NO not being able to escape the proximal heme environment, as experimentally observed, and indicate frequent contacts between dissociated NO and, amongst other residues, the histidine residue that coordinates to the heme in the deoxy form [89].

3.3. General NO rebinding properties

As can be seen from the compilation of NO rebinding kinetics measured with transient visible spectroscopy in Table 1, in the vast majority of the investigated native proteins the fastest phase of NO rebinding has a time constant of 5–8 ps. The notable exceptions that lack such a fast phase are NOS (discussed above) and heme-copper oxidases (see Section 4.1). Zemojtel and coworkers [94], in a transient infrared study of the population dynamics of the heme-bound NO in Mb mentioned a 1-ps phase in the kinetics, but this phase does not appear perceptible in the kinetic traces and was also not observed in a subsequent similar study by Kim and coworkers [95].

Because of its high speed, the "universal" 5–8 ps phase has been suggested by many workers to reflect the barrierless rebinding rate. This was indeed found to be the case in an extensive temperature dependence study on Mb and model compounds by Champion's group [96], where the rate of this phase (but not of slower phases) remained virtually unchanged down to 10 K. Also in view of the similar rebinding to 4-c and 5-c heme (Section 3.2), it was proposed that (possibly unlike for CO) Fe motions perpendicular to the heme prior to binding are not limiting the binding process. Along the same lines, the rate of the fast phase of NO rebinding was found to be independent of glycerol concentration [36,97,98].

For rebinding of a variety of intrinsic residues to the heme (Section 3.6), that can in principle not migrate far away from the heme, a rate very similar to that of the fast phase of NO rebinding is observed. This process presumably also is barrierless.

3.4. CooA

Carbon monoxide is endogenously produced by many organisms as a product of heme degradation [77] and can act as a signaling molecule [99]. A few heme-based CO-sensors have been identified [100], the best characterized being CooA [101]. CooA is found, amongst others, in the purple bacterium *Rhodospirillum rubrum*, which can use CO as a unique source of energy in the absence of oxygen. It is a transcription factor that can bind to DNA (at a DNA-binding domain) only if CO is bound to heme in a sensor domain. The homodimeric protein binds two heme groups at the interface of the subunits, in the ferrous unliganded state with histidine and proline, from two different subunits, as axial ligands [102]. In the CO-bound form CO replaces the proline residue as axial ligand [102]. It is an example of a rapidly expanding group of heme proteins that do not form a 5-c high-spin form in the steady-state, but where an external ligand can replace an internal ligand. In these proteins, the 5-c heme constitutes a signaling intermediate state for CooA that can only be generated by photodissociation of the 6th ligand. In this way, picosecond time-resolved resonance Raman (TR^3) spectroscopy has been used to characterize the CO-dissociated state [103]. In particular, the presence of an Fe–His mode in this state directly demonstrated, in agreement with prior suggestions based on steady-state spectroscopy, that CO replaces the proline axial residue and not the histidine residue. Interestingly, picosecond resolution was required for these experiments, as the 5-c state largely disappeared on the hundreds of picoseconds timescale [103], and a 5-c state could not be generated by strong steady-state illumination as in many other CO-binding heme proteins. Time resolved visible [104] and infrared [105] spectroscopic studies indeed revealed that dissociated CO rebinds to the heme for 90–98% on the sub-nanosecond timescale (Pro binding after CO dissociation occurs only on the millisecond timescale [106]). This recombination is much faster and more efficient than in many other heme proteins (in particular in Mb no rebinding takes place on this timescale and only a few percent on the hundreds of nanoseconds timescale [12]), indicating few restrictions to rebinding of dissociated CO and a tight distal pocket.

The CO geminate rebinding kinetics in CooA are biexponential, with time constants of ~70 ps (~60%) and ~400 ps (~30%) [104]. Analysis of changes in the infrared spectrum of the stretch of the heme-bound CO as a function of time suggest that the latter phase is associated with a somewhat modified protein environment and can thus be associated with a, yet to be further characterized, signaling intermediate [105].

Transient visible absorption experiments [104] indicate that an internal ligand (possibly the Pro axial residue, although this issue was not discussed) can be dissociated, and rebinds with a major phase of 6.5 ps, a timescale also observed for internal ligand rebinding for other heme proteins (see Section 3.6). A small further decay phase (6% at 440 nm) with a time constant of ~170 ps was identified and associated with a protein relaxation influencing the spectrum of the 6-c heme [104]. In view of similarity with a slow residue rebinding phase observed in the heme-based sensor Dos (Section 3.6, [107]), it would be interesting to investigate, by assessing full spectral characteristics of this phase, whether it might also reflect recombination from an intermediate 5-c species.

3.5. FixL

The bacterial heme-based oxygen sensor FixL [108] has been first described in 1991 [109]. It contains a histidine kinase domain and a heme-binding PAS domain. PAS domains are found as sensing modules involved in a wide variety of environmental signals [110]; they can contain a variety of cofactors (including flavins and coumarins) or no cofactor. Fast spectroscopic studies on FixL have been performed essentially on the isolated heme domain. One of the interesting properties of FixL is its extremely low affinity for O_2, about two orders of magnitude below that of Mb [111]; in air it is only ~80% saturated. This low affinity is in agreement with its function to detect changes between micro-aerobic and aerobic conditions associated which switching from nitrogen fixation to oxidative respiration in Rhizobia. The crystal structure of the FixL heme-domain (FixLH) has been determined in various steady-state redox and ligation forms [112–115]. Models of the pathway of signal transmission from the heme (where O_2 and other ligands bind) towards the enzymatic domain are essentially based on these structures. In particular, an important role has been proposed for a distal Arg residue (Arg220 in FixL from *Bradyrhizobium japonicum*) that makes a substantial swap between

the oxy-form, where it hydrogen bonds with the heme-bound O_2, and the deoxy form, where it makes a salt bridge with a heme propionate group [115].

The first attempt to generate a state related to a signaling intermediate by dissociation of ligands concerned a pseudo-steady-state resonance Raman study of the FixL–CO complex with nanosecond pulses [116]. Here, spectra obtained under high-intensity light indicated appearance of a (photodissociated) species with strain on the heme. Consistently, femtosecond time-resolved transient absorption measurements revealed that the absorption spectrum of the CO-dissociated species has a slightly perturbed Soret absorption with respect to the steady-state deoxy spectrum, a perturbation remaining out to the nanosecond timescale [117]. In the NO-complex, where the majority of photodissociated NO rebinds on the picosecond timescale as in Mb (Table 1), and where consequently a pseudo-steady-state photodissociated species cannot be generated, this perturbation was found to be stronger. Given that (a) such perturbations are not observed in non-signaling proteins like Mb (which has in addition very similar steady-state heme structures and spectra as FixL) and (b) the extent of the perturbation correlates with the extent of the (small) effects on the enzymatic activity of CO and NO, this has been taken as a suggestion that a signaling-related intermediate is generated in this way [117]. In both cases the spectral properties of the photogenerated state did not change up to 4 ns. Recent transient absorption [118] and X-ray crystallography [119] studies indicate that, after CO dissociation, the heme domain has adopted the steady-state deoxy configuration on the microsecond timescale.

The physiologically most relevant approach is in principle to study intermediates formed by dissociation of O_2. Generally, experiments on heme oxycomplexes are relatively complicated by the high rates of autooxidation (this can be circumvented in FixL and Mb by a suitable choice of reduction agents [117]) and the low quantum yield of photodissociation (~30% on the time scale of a few picoseconds in MbO_2 [14]). In view of the strong similarities both, between the structures of the heme-O_2 moieties and between the electronic spectra of FixLH and Mb, the photodissociation quantum yield is likely similar in both systems. A surprising result was that in FixLH, and the related DosH (see below), the quantum yield of a deoxy-like species on the tens of picosecond timescale was only ~5% [11,107,117]. A phase with a time constant of ~5 ps was observed in transient absorption experiments and initially assigned to heme-O_2 recombination from a strongly perturbed (much more than with CO and NO) form [117]. Very recent TR^3 experiments showed however that this species does not correspond to a deoxy species with a domed heme, but has to be reinterpreted as a hot 6-c heme [11], and recombination of the initially photodissociated species is probably as fast as ~80 fs. This implies that it occurs prior to, or in competition with, Fe out of plane motion, which is thought to take ~200 fs [120,121]. Thus, the distal heme pocket of the oxygen sensor acts as an effective trap for bound oxygen. Together with the low rate of bimolecular O_2 binding [111] this implies that there are high barriers for exchange between O_2 in solution and in the heme pocket. This slow exchange implies that the protein functions as a bi-stable switch which is protected to a certain extent from very rapid environmental fluctuations in both the on and off state [117], occurring on a timescale faster than the desired physiological response to change in O_2 pressure.

An ~80 fs phase such as in the FixLH–O_2 complex, is also observed in MbO_2, but with a lower amplitude [11]. This may imply that the actual O_2 photodissociation yield is higher than the value of 0.3 determined on the picosecond timescale [14].

Comparing the geminate rebinding properties of O_2 with FixLH, NO with guanylate cyclase and CO with CooA (see above), it is remarkable that, although occurring on different timescales, in all three ligand sensors rebinding specifically of the physiological ligand is extremely efficient. For all sensors, this may be considered as a mechanism to adopt the frequency in which the full intraprotein signaling conformational change is set in motion to the intrinsic heme-ligand thermal dissociation rate.

As mentioned above, for FixLH, a conformational change of distal arginine has been proposed to play a role in the signal transmission from the heme environment. Mutagenesis studies have indeed revealed strong changes in the interaction of the protein with O_2 [122,123]. Ultrafast transient absorption [124] and transient Raman [11] studies indicate a crucial role of this residue in the very fast rebinding of O_2 after dissociation: much higher yields of dissociated O_2 were observed in FixLH from *B. japonicum* in which Arg 220 had been replaced by several other amino acids. Thus Arg 220 keeps O_2 in place after dissociation enhancing the probability of reformation of the heme-O_2 bond prior to heme doming (see above). This is presumably due to the hydrogen bond between Arg 220 and O_2 already present in the bound state, but not only: in the R220H mutant, where O_2 makes a H-bond with His 220, substantially more O_2 escapes from the heme on the picosecond timescale than in the wild type protein [124].

This interpretation is supported by molecular dynamics simulations that indicate that the hydrogen bond between Arg 220 and O_2 remains intact after dissociation, and that, in contrast to mutant protein models, in most cases, dissociated O_2 remains indeed close to the binding configuration with the heme [124]. In several simulations on the wild type complex, O_2 was however seen to migrate out of the heme pocket on the timescale of ~50 ps. This motion appears a prerequisite for Arg 220 to swap in the direction of the heme propionates (with which it interacts in the switched deoxy state [115]): in some trajectories O_2 'drags' Arg 220 along by remaining in hydrogen bonding interaction ([124], Fig. 4). Although these dynamics are difficult to study experimentally due to the low yield of O_2 dissociated complexes on the picosecond timescale, they give insight in potential pathways of signal transmission and can help to guide studying these pathways indirectly using variational approaches involving genetic engineering of the protein. Indeed, whereas after Mb [28], the heme domain of FixL was the first heme protein to be studied by time resolved X-ray crystallography [119], this has yet only been possible with CO as a ligand, and essentially a configuration identical to the final deoxy state is observed. As discussed in Section 3.3, using intense nanosecond pulses, substantial amounts of O_2 can be dissociated per pulse on longer timescales. This has been exploited recently by

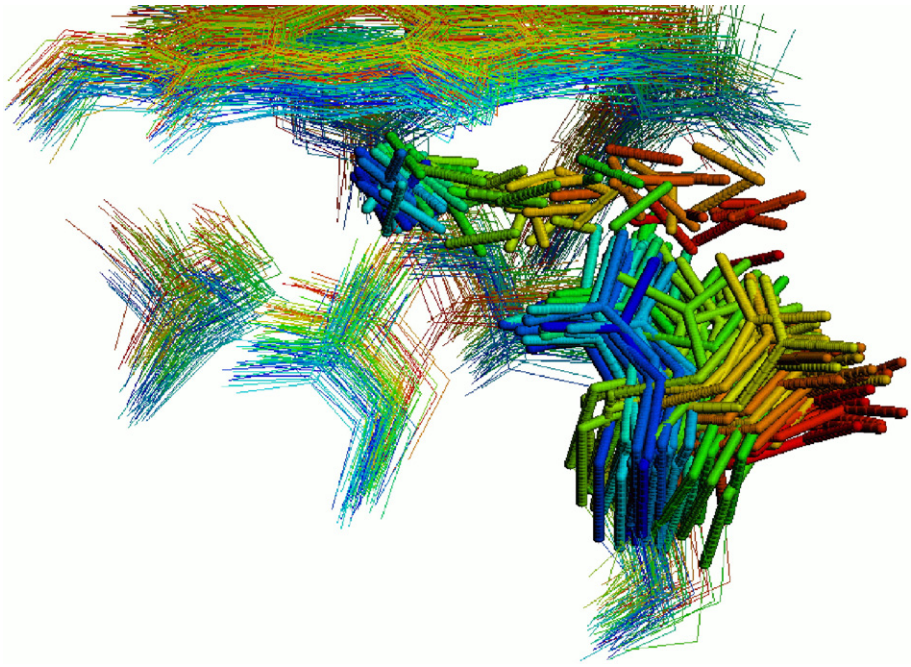

Fig. 4. Molecular dynamics of oxygen dissociation from the oxycomplex of wild type FixLH from *B. japonicum.* In most simulated trajectories dissociated O_2 remains close to the heme, favoring rapid rebinding (which is not simulated). Shown is one of the few simulation trajectories in which oxygen leaves the heme pocket. O_2 and Arg 220 are both in sticks. O_2 is initially bound to the heme. Dissociated O_2 and Arg 220 swap concertedly and remain within hydrogen bonding distance. The figure shows a superposition of structures at 1 ps intervals between −50 ps and 50 ps with respect to simulated Fe–O_2 dissociation. Blue to red color coding refers to increasing time. Reprinted with permission from Ref. [124]. Copyright (2006) American Chemical Society.

Hiruma and coworkers to monitor hydrogen bond formation between Arg 220 and the heme propionates in ~1 μs using TR^3 spectroscopy [125].

3.6. Dos

The PAS heme domain of the more recently discovered sensor protein Dos ("Direct oxygen sensor") from *Escherichia coli* displays 60% homology with FixL, but has distinct heme coordination properties [126]. Whereas in FixL the Fe^{2+} and Fe^{3+} unliganded states are 5-coordinate, in Dos they are 6-coordinate, with H_2O and Met 95 (the corresponding residue is Ile in FixL) as 6th ligands, respectively [127,128]. On this basis, and because of the influence of the heme redox state on the phosphodiesterase activity of the enzymatic domain with cyclic AMP as substrate, it has been proposed that Dos acts as a redox sensor [129] rather than, or as well as, a direct oxygen sensor as initially proposed [126]. Very recently, a role as a general gas sensor has also been proposed on the basis of moderate up-regulation of activity by CO, NO and O_2 with cyclic diGMP as substrate [130], and a crucial role of M95 in this regulation has been demonstrated [131]. In all cases, the sensing mechanism must involve a swap of the 6th heme ligand. Thus, switching of the sensor in either direction is initiated by dissociation of a ligand from the heme, and the initial processes can in principle both be studied by time-resolved spectroscopy (see Fig. 5 for a general scheme). Ultrafast absorption studies of the heme domain DosH indicate that the constraints on dissociated O_2 are very similar to those in FixLH discussed above [117]; likewise no transient Raman signals were observed on the picosecond timescale [132]. This is again in line with the observations for other ligand sensors, but prevents characterization of signaling intermediates.

Recombination measurements for both NO (Table 1) and CO indicate a relatively low ligand escape probability from the heme pocket [107]. Mutagenesis studies [98] indicate that this is not due to constraints imposed by Met 95, consistent with the notion that it is folded out of the heme pocket in complexes where it does not coordinate to heme [127,128]. The relatively efficient rebinding of CO (60% in 1.5 ns [107]), may be due to a distal heme environment allowing favorable orientation (i.e. perpendicular to the heme) for coordination to heme. For comparison, in MbCO dissociated CO remains close to the heme, but is sequestered roughly parallel to the heme plane and virtually does not rebind prior to migration out of the heme pocket (see Section 2.2). Reduced rotational hindering of dissociated CO appears to play a role in extremely efficient nanosecond CO rebinding in the truncated hemoglobin HbO from *Mycobacterium tuberculosis* (A. Jasaitis, H. Ouellet, J.-C. Lambry, J.-L. Martin, J.M. Friedman, M. Guertin & M.H.V., unpublished results).

The relatively simple CO rebinding kinetics in DosH can be described in a kinetic model by competition between rebinding to the heme (rate constant $(2.5\ ns)^{-1}$) and migration out of the heme pocket (rate constant $(3.8\ ns)^{-1}$). These properties allow a straightforward way to study the thermodynamic properties of CO motion in a heme protein on the timescale of a few nanoseconds (in Mb). A recent temperature dependence study of this competition shows that the direct CO rebinding is essentially activationless, but the motion out of the heme pocket thermally

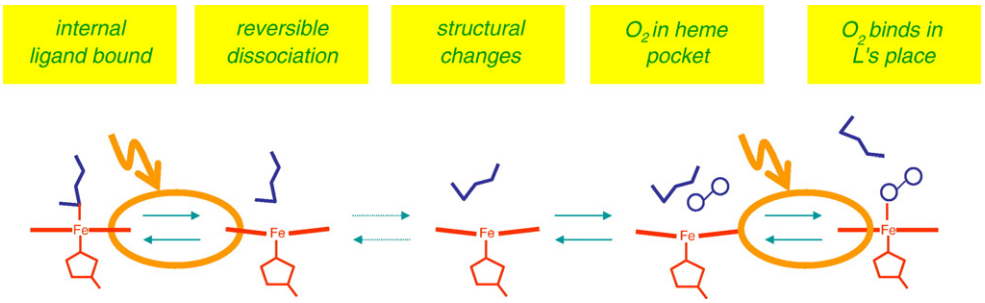

Fig. 5. General scheme of exchange between internal and external ligands at the 6th coordination position of heme iron (here methionine and O_2 as in Dos). The steps starting from both steady-state 6-c configurations (orange ellipses) can be triggered by light.

activated [98]. Thus, a clear distinction can be made between conformational changes of the protein-dissociated CO system, that involve barrier crossings [18–20] and motions within the close heme environment.

As indicated above and in Fig. 5, in Dos the initial steps of signaling by ligand exchange can also be initiated by dissociation of the internal ligand Met 95. Whereas in other 6-c heme proteins (Pro in CooA, see above, Met in cytochrome *c*, see below, and several others [133]) rebinding of a dissociated residue occurs predominantly in 5–7 ps, remarkably in Dos two distinct phases of ligand rebinding of 7 ps and 35 ps, of similar amplitude, are observed [107]. Thus, a second configuration of dissociated Met can be populated from which recombination is relatively hindered, and that can probably be associated with an intermediate in the signaling pathway that involves a substantial change in the configuration of Met 95. Indeed when Met 95 is replaced by the less flexible residue His, the slower phase disappears [98]. Whereas further progress along the switching pathway may require the presence of the external ligand close to the heme within picoseconds after Met dissociation (a requirement experimentally not readily realizable during photodissociation experiments), this very assessment gives a perspective for mapping out of the structural dynamics associated with the initial switching pathway using transient vibrational spectroscopy and simulations.

4. Oxidases

4.1. Heme-copper oxidases

Oxidases are very important examples of heme-containing enzymatic complexes. In these membrane proteins, the oxygen substrate is bound to heme in the active site and reduced to water by four electrons, while the radical and highly reactive intermediate products are maintained within the active site. CO and NO are competitive ligands that reversibly inhibit the enzyme's activity and, especially in the case of NO [134], play an established role in its regulation. In the best-characterized family of heme-copper oxidases [135–137], including the mammalian cytochrome oxidases aa_3, the active site contains heme a_3 and a nearby (~5 Å) copper atom Cu_B; one more heme *a* is present and functions as electron transfer intermediate (Fig. 6). The two metal constituents of the active site both have roles as electron carrier and as binding site for external ligands; the heme having generally a higher affinity.

4.1.1. Ligand dynamics

The presence of two well-defined close-lying binding sites gives a unique opportunity to study ligand transfer pathways inside a protein in real time. The short lifetime of the (in principle photo-dissociable) heme-O_2 complex, the direct precursor of the enzymatic reaction, precludes direct studies of O_2 transfer between heme a_3 and Cu_B. However the early dynamics upon dissociation of the CO inhibitor have been studied using various time resolved optical techniques [75,138–141]. The CO-complex contains only one CO molecule, bound to heme a_3 [142]. An early (1991) femtosecond visible absorption study [141] characterized the photophysics of the mammalian enzyme, including CO dissociation from heme a_3 in less than 100 fs and processes assigned to heme *a*. The latter include dissociation and rebinding in a few ps of the axial histidine, on a similar timescale as in the bis-histidine complex cytochrome b_5 [143].

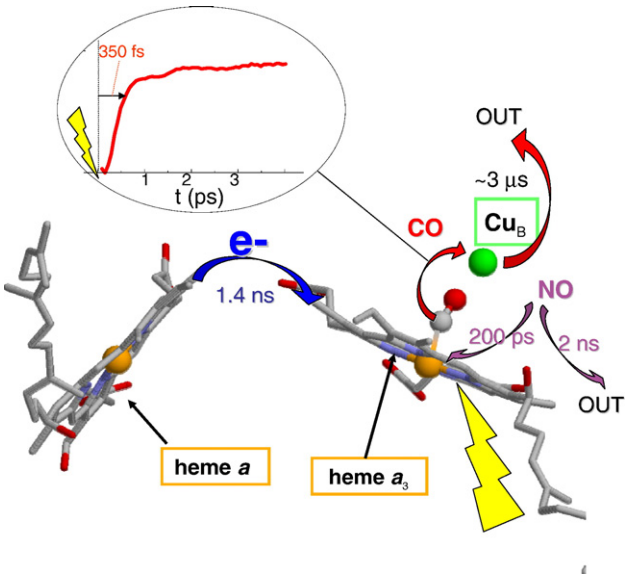

Fig. 6. Summary of processes occurring following photodissociation of ligands from various forms of cytochrome oxidase aa_3. The inset shows the formation kinetics of the ground state of unliganded heme a_3 after excitation of the CO-bound heme. See text for further details.

A 6-ps transient in this study was suggested to reflect transfer of the proximal histidine from heme a_3 to Cu_B [141], but this model later appeared inconsistent with the His-coordination of heme a_3 on the picosecond timescale subsequently observed by Raman spectroscopy [140] and with the X-ray structure [137].

For detecting Cu_B-CO bond formation, infrared absorption measurements are required. Using picosecond pulses, it was established that this bond was formed within an upper limit of 1 ps after photodissociation of the heme-CO bond [138,139], an unprecedently fast bond-breaking bond-making intraprotein reaction. In view of this result, it was suggested that the dynamics of the active site pocket constituents assist in this process, and that the reaction is not diffusion-limited, but rather ballistic. The subsequently determined X-ray structures [136,137] indeed show a very confined space, and molecular dynamics simulations indicate that rotation of the CO molecule mainly constitutes the reaction coordinate during transfer [144]. High-resolution femtosecond absorption measurements showed that the heme population dynamics (formation of the ground state of unliganded heme a_3) follow stepwise kinetics (first step ~350 fs, step length ~700 fs, Fig. 6), implying ballistic rather than stochastic dynamics [75], presumably involving low-frequency global vibrational modes, including heme doming. Very recently, using improved femtosecond infrared technology allowing full spectral measurements with ~200 fs resolution, a similar ballistic component was resolved in the formation of the Cu_B–CO bond [145]. Altogether this work provided the first evidence that functional structural dynamics can take place in a coherent regime, making use of collective protein motions. This regime contrasts with the stochastic motions occurring on timescales after energy equilibration between different modes. Although many other short distance intra-protein reactions may not be synchronizable by light on the timescale of the relevant vibrational motions, and therefore harder to assess, such reactions are likely to play a role in other systems as well.

Investigations of the interaction of NO with the active site of cytochrome oxidase are motivated by the physiological role of NO as a signaling molecule that can also regulate respiratory activity by competitive binding to the active site [134,146]. NO interacts in a quite different way as CO. At high enough NO concentration, two molecules interact with the active site, one is bound to the heme and one presumably to Cu_B. In aa_3 oxidase from *Paracoccus denitrificans*, under conditions where only one NO molecule binds, dissociation of the heme-NO bond presumably leads to efficient formation of a Cu_B–NO bond, as no rebinding to heme occurs before the tens of nanoseconds timescale [76,147] (infrared measurements of this process are still lacking as NO absorption is relatively weak). The same holds true for the ba_3 oxidase from *Thermus thermophilus*, where NO reductase activity prevents the build-up of a population with two NO molecules [147]. In aa_3 oxidase, in the presence of two NO molecules (affinity ~1 μM for the second NO), after dissociation of one NO from heme a_3 binding to Cu_B is not possible, and rebinding of ~90% NO occurs in ~200 ps [76,147] (the remaining 10% presumably escapes from the active site). Inspection of Table 1 highlights the absence of a fast rebinding phase on the 10-ps timescale that has substantial amplitude in other proteins without a nearby second binding site. This absence suggests that rebinding is in someway hindered. Indeed it is likely that the presence of the second NO congests the active site in such a way that NO binding to heme a_3 is altered: EPR measurements and molecular modelling indicate that the Cu_B-bound NO forces a rotation of the heme-bound NO away from its "free" position [147]. Recent work along similar lines on the mammalian enzyme indicates different dynamic and binding properties for both the first and second NO molecule, that may be related to different environmental constraints compared to the bacterial enzyme [148]. Altogether, picosecond NO dynamics in the active site appear to sensitively reflect the environment. Additional nanosecond geminate recombination phases also take place, presumably from NO docking sites outside of the active site [147,149].

4.1.2. Electron transfer dynamics

The combined capacity of ligand-binding and electron transfer of cytochrome oxidase aa_3 offers the unique possibility to trigger intra-protein electron transfer events by ligand dissociation. It was recognized in the late seventies [150] that backflow of electrons from the active site to the other redox centers can be induced by a midpoint potential jump following CO dissociation from the mixed valence form of the enzyme (heme a_3 and Cu_B reduced, heme a oxidized). Whereas slower equilibration steps had been studied before [151–153], only recently the intrinsic kinetics of electron equilibration between hemes a and a_3 in the mammalian enzyme was established at 1.2 ns [154]; a similar value was observed for the equivalent process in a bacterial enzyme [155]. As under turnover conditions the enzyme is often in a partially reduced state with electrons equilibrated between hemes a and a_3, fast equilibration may help to ensure optimal binding of oxygen to heme a_3, which requires heme a_3 to be reduced [154].

The equilibration time is rate-limited by the physiologically relevant forward rate of heme a_3 reduction by heme a (determined at $(1.4\ \text{ns})^{-1}$), a reaction that cannot be monitored by external electron injection, because it occurs faster than preceding reactions. This assessment provided the fastest known electron transfer reaction in a non-photosynthetic protein, and allows testing of widely varying theoretical predictions of the rate of this 'model' membrane protein reaction. In particular the observed rate is very close to a value estimated by Tan and coworkers from a pathway analysis [156]. As the driving force of the reactions involved is small and the extent of induced electron transfer limited (10–15%), the driving force can be directly determined from the amplitude, and the system provides a unique opportunity to determine the activation barrier and the reorganization energy of a ground-state electron transfer in a hydrophobic protein environment. The low value of the reorganization energy (<200 meV) found under these conditions [157] contrasts with the much higher values usually assumed for intra-protein reactions [158].

A summary of the various processes in and near the active site of cytochrome c oxidase aa_3 established from photodissociation studies on various forms of the enzyme is presented in Fig. 6.

4.2. bd oxidases

A completely different class of oxidases are *bd* oxidases [159], that have a very high affinity for oxygen, are widely spread in prokaryotes and often assure their survival under microaerobic conditions. These enzyme complexes bind three hemes; low-spin heme b_{558}, that has an electron transfer function, high-spin heme b_{559}, and 'heme' *d* (which contains an unsaturated pyrrole ring and is formally actually a chlorin), where O_2 and competing ligands bind. Although the structure of these oxidases is unknown, it has been proposed that heme b_{559}, that can also bind CO, acts as a functional analogue of Cu_B. Due to its high affinity O_2 binds to the oxidized complex to form a mixed valence–O_2 complex that cannot further react. Thus, dissociation of CO or O_2 from heme *d* may lead to transfer to heme b_{559}, a process in principle accessible by visible spectroscopy. However, O_2 dissociation appeared not to occur (at least after 1 ps) [160], possibly due to similar constraints as now established for FixL (see Section 3.5, [11]). CO dissociation does occur, but no features attributable to CO binding to heme b_{559} appeared [161]. By contrast, strong evidence for interactions between hemes *d* and b_{559} was found, as dissociation of CO from heme *d* induced complex and redox state-dependent changes in the spectrum of heme b_{559} [160,161]. Such interactions cannot be deduced from the steady-state spectrum, which is highly congested. In addition, heme *d*-CO geminate rebinding occurs on the 100-ps timescale, but only when heme b_{559} is oxidized [161]; recent studies have shown that this effect gives rise to redox control of the (much lower) ligand k_{off} rate [162]. These features imply that the two hemes are in close contact, together exert ligand guidance and probably form a di-heme active site. Using polarized femtosecond spectroscopy, boundary values for the angle between the two hemes were established [160]. Altogether, these studies provide an example of the use of ultrafast spectroscopy in predicting structural features in multi-heme proteins.

5. Cytochrome *c*

Cytochromes are redox-active proteins that do not, usually, bind external ligands. However, studying their dynamics is of interest, because a) they form a model for a heme protein that is *a priori* optimized for electron transfer and not for ligand transfer and b) modified cytochromes can bind external ligands and can sometimes be used to light-trigger electron transfer.

The hemes in native cytochromes are 6-coordinate. The first femtosecond absorption study on dynamics of cytochromes, by Traylor and co-workers [143], concerned a comparison of reduced cytochrome b_5, which is coordinated to the protein via two histidine axial bonds to the heme iron, and cytochrome *c*, which is cross-linked to the protein via two covalent bonds between cysteine residues and heme vinyls and where the heme iron is coordinated with a histidine and a methionine residue. Using UV excitation, in both cases transient spectra were observed that were assigned to 5-c species due to dissociation of a heme iron ligand, and recombination in ~7 ps. For cytochrome b_5, the dissociated residue can only be His. From a spectral comparison between the two species the authors proposed that in cytochrome *c* it is also the histidine that is dissociated [143]. This view was challenged more recently by Champion's group based on transient absorption experiments of cytochrome *c* using excitation in the visible [163]. By comparison of the photoproduct spectrum with a model compound, and interpretation of a vibrational feature in the coherent response of the system in terms of the Fe–His vibration at ~220 cm^{-1} (see Section 2.4), they proposed that it is the methionine residue that is dissociated. Recent time resolved Raman studies by our laboratory indeed monitored this band in the dissociated cytochrome *c* complex (this band is only Raman-active in 5-c heme-His complexes) [53,164]. Thus in reduced cytochrome *c*, methionine can be photodissociated with near-unity quantum yield [163], and rebinds in ~6 ps [53,143,163]. As has been alluded to previously, this rate of internal ligand rebinding appears similar for many 6-c heme proteins, with the possible exception of the signaling protein Dos (see Section 3.6).

The photophysics of the oxidized cytochrome *c* complex has also been studied with femtosecond Raman and absorption spectroscopy [53]. As in most ferric heme complexes dissociation of the internal axial ligands appears not to occur in this case; vibrational relaxation of the heme does occur on the time scale of ~7 ps (see Section 3.3). In this context, we did previously suggest that a minor phase in the photophysics of the ferric heme domain of Dos was due to dissociation and rebinding (in ~20 ps) of a ligand [117], which was then thought to be methionine. The later-established structure of this domain showed that this ligand is H_2O [127,128].

It is thought that cytochrome *c* functional events do not include binding of external ligands. A possible exception is nitrosylation of mitochondrial cytochrome *c* during apoptosis [165]. Formation of a native ferrous cytochrome *c*–NO complex is possible, but requires prolonged equilibration with 1 atm NO at relatively low pH. Once formed, this complex is extremely stable. Indeed photodissociated NO fully rebinds in ~8 ps [90]. The lack of further decay phases is consistent with a rigid protein environment that does not allow dissociated NO to adopt different configurations prior to rebinding.

The cytochrome *c*–CO complex cannot be formed from the native protein. However, in cytochrome *c* where the Met heme ligand is chemically modified or replaced by genetic engineering, CO does bind [166–168]. Such proteins are employed as near-native photo-activatible electron donors [167,168], however geminate recombination of heme-CO pairs on the timescale faster than intermolecular electron transfer (typically microseconds) limits the yield of this process.

Recent investigations of this process with ultrafast spectroscopy have shown that the rebinding kinetics of CO are highly multiphasic on the 10^1–10^4 ps timescale, and extremely sensitive to the local heme environment [91,169]. Thus CO rebinding in cytochrome *c* can be seen as an equivalent to NO rebinding to Mb, in that it is a sensitive probe of the properties of the heme cavity (see Section 3.3). Interestingly the highest yields (virtually 100%) of CO geminate rebinding to cytochrome *c* (and to any heme protein) are obtained when a small residue like Ala replaces Met. In this case CO can adapt in the

cavity without much steric interference and, after dissociation, is maintained in place and in the right orientation to rebind efficiently; partial rebinding phases as fast as 14 ps are observed [91]. This is faster than in any other heme protein, and it approaches the barrierless NO rebinding rates (Table 1). Replacing Met with larger and charged residues, like Arg, allows a substantial fraction (~25%) of dissociated CO to escape, presumably by opening up the cavity. Such studies help in guiding optimization strategies for CO escape. In a more general context, the high geminate recombination rates and the corresponding rigidity of the heme pocket are in agreement with the electron transfer function of the protein, which should allow optimal performance with minimal structural fluctuations.

6. Outlook

Whereas initial studies on the dynamics of heme proteins were essentially limited to Mb and Hb in combination with the inhibitors CO and NO, during the last decade a much wider variety of proteins has been investigated using ultrafast techniques, in particular in interaction with their physiological ligands. The systems reviewed here (doubtlessly biased by the author's present research interests) thus start to constitute a reference framework for the large variety of fast functional ligand, protein backbone, and electron dynamics possible within heme proteins. Along with the availability of high-resolution static structures, characterization of identified heme proteins will benefit from the presently accumulated reference body. A particular example is constituted by the rapidly increasing number of heme-containing PAS domain sensors [110]. On the other side, maturation of new ultrafast techniques, including for instance multidimensional infrared techniques and femtosecond X-ray crystallography, will allow to obtain more detailed dynamic pictures, in particular from contributions to functional events of the protein moiety of heme proteins.

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