Unravelling the "transition-state" of the respiratory function

Ever since the end of the 19th century, light has been used to mimic and probe the detachment and rebinding of ligands to the Iron atom of the porphyrin in haem proteins, such as haemoglobin (Hb) and myoglobin (Mb). Haemoglobin is a tetramer (Fig. 1(a)) of four myoglobin-like (Fig. 1(b)) units. The most important ligand for respiration is of course molecular oxygen, but Mb and Hb can also bind small ligands such as CO, NO, H_2O, CN, etc., which confer them different biological functions. For example, NO is well-known for neurotransmission, regulation of vasodilatation, platelet aggregation, and immune response in our organism.

The process of ligand binding and release is accompanied by a significant structural and electronic change of the haem porphyrin. It is well established since the works of Pauling *et al.*, that when the ligand is bound, the porphyrin (Fig. 1(c)) is in a low spin (LS) state (Fig. 1(d)) and is planar, while without ligand (the so-called deoxy form), the porphyrin is in a high spin (HS) state and is domed with the Fe atom moving out of the porphyrin plane on the proximal side (Fig. 1(c)). This in-and-out motion of the Fe atom is the trigger of large-scale motions of the entire protein, which lead to the so-called tense (S) and relaxed (R) state of haemoglobin that is essential in respiration.

With the birth of femtosecond (fs) spectroscopy in the 1980s, the first systems to be investigated were actually haem proteins with the aim to describe in "real-time" the process of ligand detachment and possibly, its recombination to the haem [1]. The methods used were pump-probe spectroscopy in the infrared and visible range [2], or time-resolved resonance Raman spectroscopy [3]. More recently, picosecond [4] and fs [5] X-ray absorption spectroscopy (XAS) were also implemented. The focus of these studies was the initial events after excitation of the haem (into the π-π^* transitions of the porphyrin) and ligand detachment, and how the system reaches the HS deoxy state. After nearly thirty years of experiments, two main interpretations have prevailed: a) formation of the HS state goes via a cascade through different electronic states [6] or; b) the excited system decays directly into the HS ground state of the deoxy form, which then undergoes vibrational cooling [2]. The first hypothesis does not exclude vibrational relaxation within the electronic states. The main difficulty in this debate stems from the fact that none of the used optical spectroscopic probes is sensitive to the electronic structure.

In order to solve this issue, we implemented femtosecond Fe K_α and K_β X-ray emission

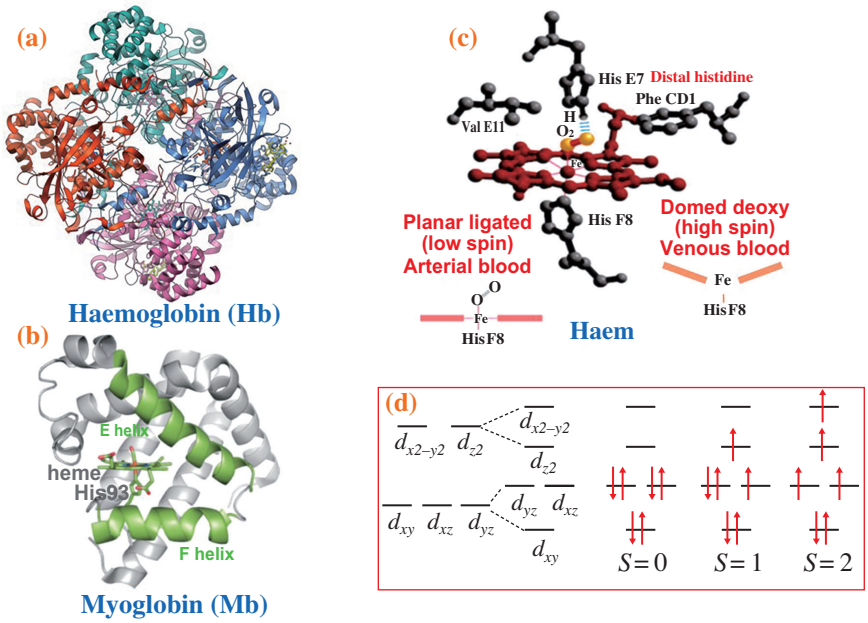

Fig. 1. **(a)** Haemoglobin with its four myoglobin-like **(b)** units. **(c)** Haem Iron-porphyrin with a ligand on the distal side and a histidine (His93, see also (b)) on the proximal side, which anchors the haem to the rest of the protein scaffold via the F-helix. **(d)** Occupancy of Fe 3*d*-orbitals that are split by the ligand-field between bonding (d_{xy}, d_{yz}, d_{xz}) and antibonding (d_{x2-y2}, d_{z2}) orbitals, for the different spin states of a ferrous haem.

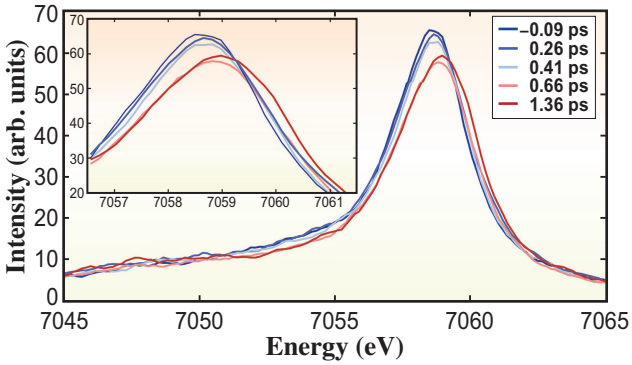

Fig. 2. Laser-off (unpumped) and Laser-on (pumped) K_β XES spectra of MbNO at different time delays between –0.09 and 1.36 ps (from blue to red) showing a blue shift of the $K_{\beta1,3}$ line and an intensity decrease. The inset zooms into the region of the maximum of $K_{\beta1,3}$ XES line showing peak shifts smaller than the energy resolution (~0.5 eV) of our experiment.

spectroscopy (fs-XES) and fs-XAS in the study of myoglobin-NO (MbNO). The choice of fs-XES is motivated by its well-established sensitivity to spin states, and the choice of MbNO is motivated by the fact that the detachment-recombination cycle takes place in ~200 ps, while all other Myoglobins have either very long (MbCO) or very short (MbCN, MbH_2O, MbO_2) cycles. Fs-XAS helps probe the structural changes [7]. Figure 2 shows a series of laser-off and laser-on K_β XES spectra at various time delays. The shift of these lines reflects the changes undergone by the system between its LS planar form and its HS domed form. The temporal evolution of the K_β line shift and the K_α line width reflects the same processes and shows a rise time of ~700 fs, followed by a biexponential decay of 30 ps and >1 ns [8]. These time scales reflect changes in the spin state of the system and cannot be attributed to thermal effects, which do not affect the XES lines. Combining these data, with our previous ultrafast optical [9], ps XAS [4] studies and the resonance Raman studies by Kruglik *et al.* [3], we deduce the scheme that is shown in Fig. 3: a) upon photoexcitation of the haem, NO dissociation is prompt and simultaneous to the passage into an intermediate spin state ($S = 1$). This first event occurs in <100 fs; b) it is followed by a relaxation to the HS ($S = 2$) state in ~600–800 fs, which leads to formation of a deoxyMb ground state; c) the rebinding of NO takes places in a bimodal mode of a few ps (for ligands in the haem pocket) and 150–200 ps for ligands that have escaped the pocket. Contrary to other ligands, NO can bind to deoxyMb and the subsequent relaxation back to a planar LS MbNO takes place in ~30 ps. Thus, the whole cycle from ligand detachment to its rebinding and reformation of a planar MbNO is a sequence of spin cross-over (SCO) and reverse SCO. It should be stressed that steps (a) and (b) are common to all Mb's undergoing ligand detachment. Only step (c) is ligand specific. Because the initial events of ligand detachment and relaxation to the deoxy form are crucial for the subsequent processes they can be considered the "transition state" of the respiratory function. However, a recent study on ferric Cytochrome c [10] shows that such spin transitions also occur in non-respiratory proteins, which calls for elucidation of their role in such systems. This work was performed at SACLA **BL3**.

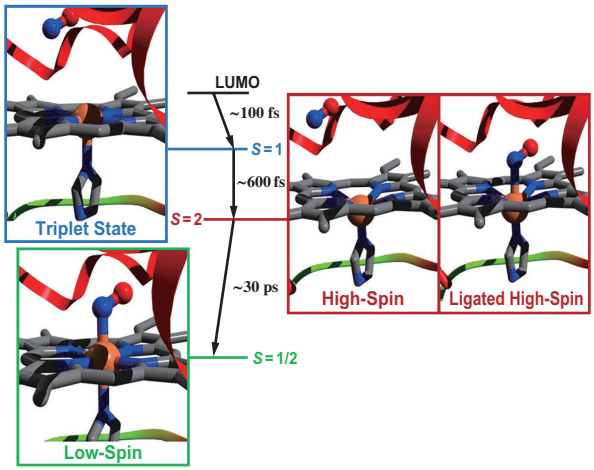

Fig. 3. Photocycle of MbNO as deduced for Ref. 8. Excitation of the Q-band of the porphyrin (π-π^* transition) leads to a prompt dissociation of NO and formation of the intermediate $S=1$ state of the deoxyMb pentacoordinated haem. This state then decays to the high spin $S=2$ state, which returns to the initial low spin state in ~30 ps after rebinding the NO ligand.

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