

Ligand discrimination of myoglobin in solution: an iron L-edge X-ray absorption study of the active centre†‡

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Iron L-edge X-ray absorption spectra of the active centre of myoglobin in the met-form, in the reduced form and upon ligation to O₂, CO, NO and CN are presented. The strength of ligation with the iron centre is finger-printed through the variation of the L₃:L₂ intensity ratio. Charge Transfer Multiplet calculations are performed and give qualitative information about oxidation states as well as charge transfer.

Myoglobin is a metalloprotein found in muscle cells, where it is responsible for the storage and the transport of oxygen.^{1,2} As the simplest globin it serves as a model system for investigating the relationship between structure, dynamics and function of the ligand binding reaction.^{1,3,4} The binding of the myoglobin (Mb) to ligands takes place at its active centre, a heme prosthetic group, consisting of an iron atom which is fourfold coordinated to a porphyrin ring. On the proximal side of the heme an imidazole group serves as an axial ligand (proximal histidine). *Via* the sixth coordination site of the heme iron, ligands like O₂, CO, NO or CN can bind reversibly to the active centre. Their binding affinity is influenced by a distal histidine (His64) which can form *e.g.* in the case of O₂-binding stabilizing hydrogen bonds to the FeO₂ complex.⁵ A wide range of studies have been carried out in order to understand the discrimination mechanism for different ligands by the Mb active centre.⁶

Here we investigate the electronic structure of the iron active centre of Mb in solution upon ligation with CO, CN, O₂, and NO as well as in comparison to the deoxidized form and in the met-form (schematic presented in Fig. 1). The electronic structure is

revealed at the L-edge of iron *via* total fluorescence yield (TFY) X-ray absorption spectroscopy (XAS), which allows the molecular orbital structure of the d-orbitals to be probed. Such valence d-orbitals of the iron are involved in bond-building and breaking as well as charge transfer with ligand.^{7–9} Note that TFY spectra of a concentrated sample suffer normally from saturation effects,¹⁰ even for concentrations down to 500 mM.^{11,12} Nevertheless, this effect is negligible in this study for two reasons; first, the measured concentration is 2 mM and second, the concentration was preserved for all measurements. The L-edge XAS for 3d-transition metals (*e.g.* iron) lies in the soft X-ray regime. This is the reason why most previous soft X-ray studies on proteins were carried out on solid, hydrated or frozen samples.^{9,13} Just recent spectroscopic developments also allowed the investigation of proteins in solution.^{7–9,14} For this, samples are separated from the vacuum by Si₃N₄ membranes of 150 nm thickness, which allow the soft X-rays to pass. In order to prevent X-ray induced sample damage the sample solution was constantly flowed through the sample flow-cell (schematic presented in Fig. 1). The samples have been prepared as shown previously.^{13,15–17} Their oxidation states and ligation were monitored *via* UV-Vis spectroscopy. As shown before by Harada *et al.*¹⁸ and Chergui and co-workers,¹⁷ such control is sufficient enough to guarantee the proper preparation of the sample, namely a +II oxidation state for the central iron atom in deoxyMb, MbO₂, MbNO and MbCO but a +III oxidation state in metMb and MbCN. Note that we cannot exclude the presence of a small Fe(II) fraction (*i.e.* deoxyMb fraction) in metMb and MbCN. However, it should be negligible based on our UV-vis spectra (see Fig. S1, ESI†). Especially the Q-band of the UV-Vis spectra is sensitive to the oxidation state and even small changes would alter it markedly.^{19,20} Details of sample preparation and ligand control using UV-Vis are described in ESI.†

In Fig. 2 iron L-edge TFY-XA spectra of Mb with different ligands are presented together with the reduced and the met-form. The spectra are normalized to the L₂ intensity. This normalization was chosen, since it was previously shown that the L₃ intensity is more sensitive to changes in the local electronic structure than in the L₂ intensity.^{21,22} Among Mb-Fe²⁺ proteins,

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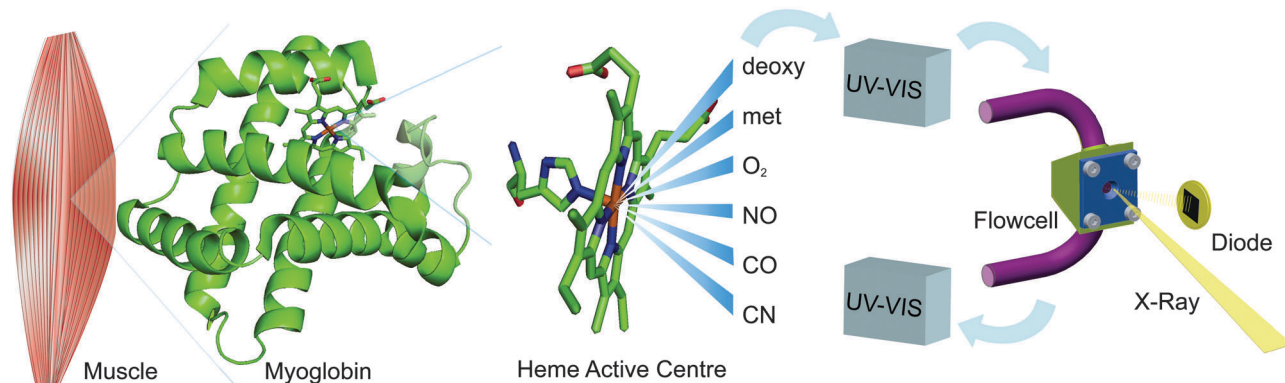


Fig. 1 X-ray absorption measurements were carried out on myoglobin, the oxygen transport and storage protein of the muscle cells, for six different ligation states of the active centre (deoxyMb, metMb, MbO₂, MbNO, MbCO, MbCN). The ligation stability of the sample solutions was monitored before and after X-ray irradiation via UV-Vis spectroscopy.

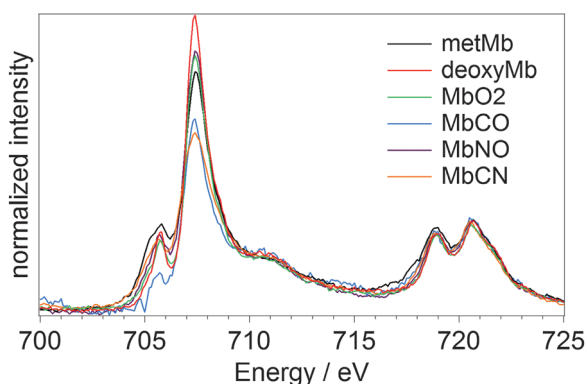


Fig. 2 Fe L-edge X-ray absorption spectra of myoglobin in various ligand configurations (deoxyMb, metMb, MbO₂, MbNO, MbCO, MbCN). The spectra were normalized to the L₂-edge intensity.

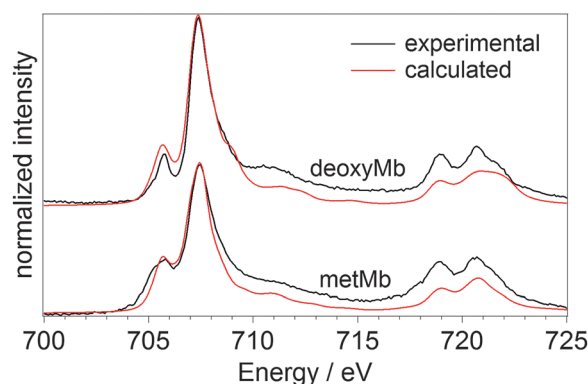


Fig. 3 Fe L-edge X-ray absorption spectra of metMb and deoxyMb from Fig. 2 compared with spectra calculated using CTM theory. Calculated spectra were normalized to each other with respect to their L₂-edge intensity.

deoxyMb leads to the highest L₃ intensity. Upon ligation with O₂, NO and CO, this intensity decreases. It looks comparable for MbNO and MbO₂ and reaches its lower value for MbCO. Among Mb-Fe³⁺ proteins, metMb shows the highest L₃ intensity, which is dramatically reduced upon ligation to CN. Such reduction of the L₃ intensity in TFY-XA spectra at the L-edge of 3-d transition metals in aqueous solution has recently been correlated with ultrafast charge-transfer processes to the solvent.^{23–25} Orbital mixing leads to electron delocalization between the valence d-orbitals of the transition metal and the valence bands of the solvent molecules, quenching some of the metal X-ray fluorescence bands.²⁶ It was argued that electron delocalization which contributes to the non-radiative process is faster than L₃ core-hole life time (2 fs)¹⁴ and slower than the L₂ one (0.7 fs),¹⁴ affecting accordingly mainly the L₃-edge.²³ In the current study, the strength of orbital hybridization between the metal active centre and the ligand reflects directly on the L₃:L₂ intensity. Indeed, this observation is similar to the recent observation by Carniato *et al.*,²⁷ where they show that the L₃:L₂ ratio is directly correlated with the strength of the chemical bond. According to the earlier observation,^{21,23,25} the reduction of L₃ intensity relative to L₂ is due to stronger interaction between the ligand and the iron centre and the

increase in the strength of orbital hybridization causes an increase in the non-radiative channel under L₃ reducing its radiative intensity relative to L₂.²³ Note that the analysis of TFY-XA spectra from transition-metal aqueous solution is complicated due to competing signal contributions from solute and solvent, causing sub-background intensity.²⁸ In the current study, therefore, the concentration of the solute (myoglobin) *versus* solvent (water) was kept constant for all samples in order to ensure that the relative changes are not caused by background effects.^{21,22}

In Fig. 3 iron L-edge XA spectra of metMb and deoxyMb calculated using Charge Transfer Multiplet Theory (CTMT), which includes ligand-to-metal charge transfer (LMCT) and metal-to-ligand charge transfer (MLCT) but does not account for the aforementioned fluorescence quenching, are plotted (see ESI† for more details). The good agreement between experimental and theoretical XA spectra suggests that the L₃ of the TFY-XAS in metMb and deoxyMb is little affected by non-radiative channels. Therefore, these proteins can be considered as benchmark molecules for further interpretation of Mb-Fe³⁺ and Mb-Fe²⁺ samples, respectively. For other ligated proteins, we could not reproduce the intensity decrease at the L₃-edge (Fig. 2) and leave unaltered intensity and lineshape at the L₂-edge,

which can be ascribed to the occurrence of fluorescence quenching in the presence of O₂, NO, CO and CN ligands.

In the frame of our CTMT calculations, the ground state configurations are 6.7% d⁴L⁻ + 65.0% d⁵ + 28.3% d⁶L⁺ for metMb and 94.3% d⁵L⁻ + 5.6% d⁶ + 0.1% d⁷L⁺ for deoxyMb, where d and L⁻/L⁺ are related to metal orbitals and an electron/a hole localized on the ligand, respectively. These configurations are in line with the expected +III oxidation state for metMb and +II oxidation state for deoxyMb. The low MLCT contribution (<10% d⁴L⁻) for metMb is congruent with former observations in the met-form of haemoglobin⁷ and some model Fe heme complexes.²⁹ In contrast, the significant MLCT (d⁵L⁻) contribution for deoxyMb is quite unusual when compared with literature data about solid samples.²⁹ This hints that, in this protein, an electron is almost fully localized to the water ligand and that the observed XA spectrum is mainly due to the protein solvation. Likewise, for MbO₂ and MbNO, the limited intensity decrease at the L₃-edge compared with the case of deoxyMb supports a proposal by Weiss³⁰ (originally for haemoglobin) that the ligation process produces Mb-Fe³⁺-like species bound to negatively charged O₂⁻ and NO⁻ ligands, respectively. In fact, TFY measurements are seemingly essentially sensitive to the large MLCT to these ligands, which might explain the similarity of the corresponding XA spectra although NO is described in the literature as a much stronger ligand than O₂.²⁷

For MbCO and MbCN, a strong fluorescence quenching happens which reflects additional charge transfers. For MbCN in particular, an increase in MLCT might contribute to lower the intensity of the most prominent spectral feature at the L₃-edge (Fig. S2 in ESI†). From the decreasing L₃:L₂ intensity ratio in Fig. 2, we can sort ligands with respect to the increasing strength of their interaction (involving fluorescence quenching) with the iron active centre of Mb, namely CN ≈ CO > O₂ ≈ NO.

In brief we have presented here the first study of ligand discrimination by myoglobin in solution using TFY L-edge soft X-ray absorption spectroscopy at the iron active centre. Based on experimental observation, we could qualitatively show that the strength of the interaction between the ligand and the porphyrin active centre can be sorted in the following order: CN ≈ CO > O₂ ≈ NO. More detailed investigations into this issue are currently in preparation using high resolution resonant inelastic X-ray scattering (RIXS) which has been introduced recently in the liquid micro-jet technique.³¹

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