

# Ultrafast 2D-IR spectroscopy of nitrosylated haem-proteins using ULTRA

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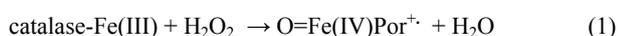
## Introduction

Understanding the function of biological molecules at the level of movements of atoms or the making/breaking of chemical bonds offers considerable potential for downstream benefits. These range from advanced drug design strategies to the production of synthetic, biology-inspired molecules for technological or medical applications. The concept of the structure-function relationship is well-established in biology but this does not offer a complete picture of the intimate 'chemical' processes occurring in the active sites of biological molecules because it neglects the role of solvent-induced and thermal fluctuations of the protein architecture as well as the effect of local vibrational modes. Indeed, the influence of fast protein structural dynamics on biological processes that take place many orders of magnitude more slowly is one of the key questions yet to be conclusively addressed.<sup>1</sup>

The subtle way that biomolecule structure influences function is evidenced very clearly by the haemoprotein family. This group of proteins are responsible for a large number of biological roles ranging from reversible ligand binding to enzymatic activity but, to a first approximation, some of the major structural features located near the haem centre appear to be very similar, raising the question of exactly how the molecular architecture influences function. An example of this can be seen in studies showing that mutations at just four positions or fewer can engender nitric oxide reductase or peroxidase activity upon the ligand binding protein myoglobin. This flexibility of function within a relatively restricted structure has led to the haemoproteins becoming attractive templates for synthetic systems but for this to be successful, we must first fully understand the detailed roles of each of the main structural elements.<sup>2-5</sup>

Ultrafast 2D-IR spectroscopy has shown great potential for measuring the structural dynamics of biological molecules both at the global, whole molecule level and in terms of a single bond within the macromolecular structure by employing vibrational probes.<sup>6-8</sup> The purpose of this report is to summarise recent advances of 2D-IR spectroscopy of haem proteins using the ULTRA laser system and to demonstrate how this technology can influence our view of the structure-function relationship. This will be done by reference to studies of two haem proteins: the ligand transport protein myoglobin<sup>9</sup> and the catalase enzyme.<sup>10</sup>

The catalases, common to almost all aerobically-respiring organisms, are responsible for the disproportionation of hydrogen peroxide in a reaction that is often represented as:<sup>11-13</sup>



where O=Fe(IV)Por<sup>+</sup> is referred to as Compound 1 (CpdI). This mechanism is widely accepted but the precise roles of catalase

structural elements in the individual steps remain the topic of debate<sup>14,15</sup> and enquiry. In particular, the distal side of the haem pocket includes a histidine residue located in close proximity to the haem centre.<sup>16-18</sup> This residue is widely implicated in the catalase mechanism and mutation studies have shown that its presence is crucial to CpdI formation.<sup>19-21</sup>

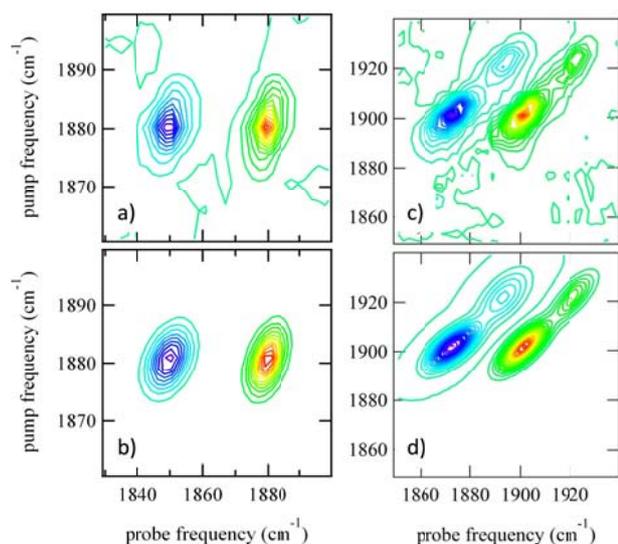
Interestingly, a similarly-located and conserved distal histidine residue is found in myoglobin. The fact that this residue is apparently central to the functioning of two different proteins begs questions about its role. For example, it seems reasonable that it could be responsible for ligand binding in both cases. This then suggests that it is the rest of the haem pocket that controls specific functionality. Other residues in the active sites of these proteins do differ and so must contribute to the behaviour of the biomolecule. Most notably, the proximal residues that coordinate with the Fe atom of the haem moiety are different and this change could play a role in the chemical lability of the haem ligand.<sup>22</sup> However, it is also instructive to ask whether the presence of the distal histidine in both myoglobin and catalase means that the haem ligand is subject to a similar chemical environment in both cases and it is this question that we address here.

In each of the articles featured, the ferric form of the protein was considered with nitric oxide bound to the haem centre acting as a probe of the local dynamic environment.<sup>9,10</sup> The choice of NO arose because it binds effectively to the haem site of Mb while the catalase enzyme is inhibited by NO binding, meaning that it provided a stable and effective probe in both cases. In addition, NO itself plays a fundamental role in biology, participating in processes such as signalling and immune responses,<sup>23-25</sup> while higher concentrations can lead to the deleterious effects associated with nitrosative stress. The NO radical is also highly reactive with transition metals and metalloproteins, such as those containing haem groups and well-known examples include components of the respiratory chain such as cytochrome C oxidase and key enzymes of the tricarboxylic acid cycle such as fumarase and aconitase.<sup>26,27</sup>

## Experimental

For all 2D-IR experiments, catalase and myoglobin were contained in a pD7 deuterated phosphate buffer solution with care taken to ensure complete H/D exchange in all cases. MAHMA NONOate was used to nitrosylate the ferric proteins.<sup>10</sup> For all 2D-IR experiments, the samples were held between two CaF<sub>2</sub> windows separated by a 100 μm thickness spacer.

The method for obtaining IR pump-probe and 2D-IR spectra has been described previously; briefly, 2D-IR spectra were acquired using the FT-2D-IR method described in Ref<sup>10</sup> using a sequence of three mid-infrared (IR) laser pulses arranged in a pseudo pump-probe beam geometry.<sup>28,29</sup> The pulses were generated by the ULTRA Ti:sapphire laser system pumping a white-light seeded optical parametric amplifier (OPA) equipped



**Fig 1:** 2D-IR spectra of the NO stretching vibrational mode of nitrosylated catalase (a) and myoglobin H64Q (c). Figs (b) and (d) are fits of the data in (a) and (c) respectively to 2D Gaussian lineshape functions.<sup>9,10</sup>

with difference frequency mixing of the signal and idler. Mid IR pulses with a temporal duration of  $\sim 100$  fs; a central frequency of  $1900\text{ cm}^{-1}$  with a bandwidth of  $>300\text{ cm}^{-1}$  were employed.

## Results and Discussion

Representative 2D-IR spectra for catalase and the myoglobin (Mb) H64Q mutant are shown in Fig 1(a-d). In the case of the catalase protein, a single negative (red) peak on the spectrum diagonal was observed and assigned to the bleach and stimulated emission from the  $\nu=0-1$  transition of the NO stretching mode of the nitrosylated protein (Fig 1 (a)).<sup>10</sup> This was accompanied by a positive (blue) peak shifted by around  $30\text{ cm}^{-1}$  to lower probe frequency, which was assigned to the accompanying  $\nu=1-2$  excited state absorption.<sup>10</sup>

The 2D-IR spectrum of wild type (wt) Mb reported was similar to that of catalase in that it too featured a single diagonal infrared transition in the NO stretching region.<sup>9</sup> By contrast, the 2D-IR spectrum of Mb-H64Q shows two diagonal peaks (Fig 1(c)), one of which was located at the same frequency as that of the wild-type protein peak and one that was shifted to lower frequency.<sup>9</sup> The structure of Mb is well-known and it is accepted that wt-Mb features a direct H-bonding-type interaction between the distal histidine residue side chain and the haem ligand.<sup>30</sup> The H64Q mutation features replacement of the distal histidine residue with glutamine. The latter features a more flexible side chain than histidine, allowing the terminal functional group of the side chain to move away from the haem ligand in a fraction of the molecules in the sample. As a result of this, the higher frequency peak observed in the Mb-H64Q spectrum was attributed to the NO stretching vibration of the sub-ensemble in which there was an interaction between the NO ligand, similar to the wild type protein. The lower frequency mode corresponded to the sub-ensemble of proteins without this interaction.<sup>31</sup>

It is noticeable in the 2D-IR spectra shown in Fig 1 that the lineshapes of the  $\nu=0-1$  and  $\nu=1-2$  transitions are elongated towards the diagonal of the spectrum. The spectra for catalase and Mb-H64Q shown were obtained with a waiting time of  $\sim 1$  ps and this elongation is due to inhomogeneous broadening of the NO stretching vibrational mode of both proteins. This effect has been widely reported for haem proteins and arises from fluctuations of the electrostatic environment of the ligand due to motion of the protein architecture.<sup>9,10,32-34</sup> As the protein fluctuates, the effect is to vary the NO stretching frequency by a

small amount leading to broadening of the transition across the ensemble. In a 2D-IR experiment at waiting times that are short in relation to the protein dynamics that are causing the broadening, this results in a diagonal elongation of the 2D-IR peaks because the sample maintains a ‘memory’ of the state in which it was excited; leading to a correlated 2D peakshape. As the waiting time is allowed to increase and becomes comparable to the timescales of the underlying dynamics, the sample fluctuations lead to a loss of this memory and the peak becomes more circular. This so-called spectral diffusion results in a change in the profile of the 2D-IR peak with waiting time and quantification of the lineshape evolution using fitting to 2D Gaussian lineshapes (eg Fig 1(b&d))<sup>9,10</sup> gives rise to an exponential-type decay with the timescales reporting the dynamics of the frequency-frequency correlation function (FFCF) of the NO vibration, which in turn report on the protein dynamics influencing the ligand.<sup>9,35-39</sup>

The FFCFs extracted from the 2D-IR data for catalase, wt-Mb and Mb-H64Q are shown in Fig 2.<sup>9,10</sup> In each case an exponential function was shown to represent the data well (solid lines) and it is interesting to note the similarities and differences in the data across the three proteins. Specifically, both catalase and the wt-Mb showed a fast dynamic component ( $\sim 3$  ps) alongside a static offset. The static parameter was assigned to slow motions causing broadening of the NO transition but which were too slow to be captured by a 2D-IR experiment that was temporally limited by the vibrational lifetime of the NO stretching vibration. Although catalase and wt-Mb showed similar fast dynamics, the static component in Mb was large, whereas this was very small in catalase. This showed that the motions causing broadening are complete within 20-30 ps in catalase while those in Mb persist for much longer. This observation was used to infer a dynamically more constricted structure in catalase.<sup>10</sup>

In assigning the fast dynamics, it was noted that while the wt-Mb and the H64Q mutation both showed similar slow/static dynamics, the fast component in the wt-Mb data was replicated only for the sub-ensemble of the H64Q mutation with a similar vibrational frequency to the wt protein.<sup>9</sup> This fast component was absent in the sub-ensemble of Mb-H64Q that had no direct link between the distal side chain and the NO ligand. This allowed assignment of the fast dynamics of wt-Mb to interaction between the distal histidine and the haem ligand.<sup>9</sup>

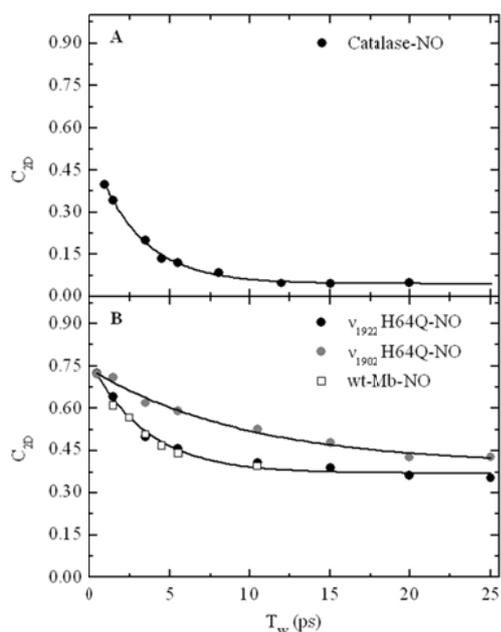
Given the similarities in both distal pocket architecture and fast dynamics between wt-Mb and catalase, an analogous assignment of the fast dynamics of catalase to an interaction between the distal histidine and the NO would seem appropriate. However, it was reported that the crystal structure of the nitrosylated protein was more consistent with an interaction of NO with a conserved bound water molecule in the distal pocket. While this would seem contradictory, the bound water molecule was also hydrogen-bonded to the distal histidine in such a way as to communicate the fast dynamics from the protein scaffold to the haem ligand. Thus, the dynamics observed in catalase were comparable to those of wt-Mb but the mechanism by which they were observed was different for the two proteins. It was further hypothesised that this bound water molecule, in conjunction with a network of others observed in the crystal structure, were the origins of the structurally more confined active site and so the small size of the static parameter in the catalase FFCF.<sup>10</sup>

In summary, by comparing these sets of results, it can be seen that 2D-IR provides insight into the local chemical environment of the haem ligand in both a ligand transport protein and an enzyme. Furthermore, cross-disciplinary interaction with structural biologists enables this data to be interpreted in a physically-meaningful manner for biological applications. The conclusions suggest that the ligand transport protein features a more flexible structure with an interaction between the distal pocket and ligand that presumably serves to aid reversible

binding of a diatomic ligand. In contrast, the enzyme locates the ligand in a more constrained geometry consistent with the need to access a particular transition state as part of the reaction mechanism. Thus, although the structures may seem similar, the natures of the active sites of these two proteins differ markedly.

#### Acknowledgements

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**Fig 2:** FFCF data for nitrosylated catalase (a) and myoglobin (b) extracted from 2D-IR data.<sup>9,10</sup> Figure reproduced from Ref<sup>9</sup> - Reproduced by permission of the PCCP Owner Societies

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