Casting a cold eye over myoglobin

Steven G. Boxer[1] and Philip A. Aufinrud[2]

Structural studies of carbonmonoxy myoglobin photolyzed at ultra-low temperatures have allowed the visualization of an otherwise elusive binding intermediate.

[1]Department of Chemistry, Stanford University, Stanford, California 94305-5080, USA
[2]Department of Chemistry, Harvard University, Cambridge, Massachusetts, 02138 USA

Two recent X-ray crystallographic studies describe the location of carbon monoxide in myoglobin (Mb) following its photodissociation from the haem iron[1,2]. This photochemical intermediate, denoted Mb*CO, is short-lived under physiological conditions and leads to conformational relaxation of the protein as well as escape of the CO. By performing the X-ray analysis at temperatures below 40 K, the investigators were able to 'freeze' conformational relaxation and trap the CO in a well-defined site near the haem. Because Mb*CO is the putative intermediate for the ligand binding process Mb + CO → MbCO, it is a key player in the story of how Mb reversibly binds ligands (Fig. 1). The authors have discovered that the CO of Mb*CO occupies a well-defined site near the haem iron with its bond axis oriented approximately parallel to the haem plane.

Why should anyone care? After all, Mb is never exposed to light in its physiological environment, CO is not its physiological ligand, and cryogenic temperatures are of no consequence for normal biology! Furthermore, Mb was the first moderately large protein to be characterized by X-ray crystallography over 30 years ago, so isn't everything that anyone would possibly want to know about it already known? The answer to this question is unambiguously no. To understand both the relevance of this seemingly non-physiological result to Mb's physiological function and the more general issues regarding the coupling between protein motion and function (issues which presently attract the interest of many scientists in different fields), a bit of background is needed.

A bit of background

Firstly, carbon monoxide is indeed an important, albeit very dangerous, physiological ligand for Mb. In the course of the natural breakdown of haem itself, a single molecule of CO is liberated The equilibrium binding constant for CO is much larger than for oxygen, but not so much larger that our Mb is saturated with CO. It is clear that the protein takes an active role in discriminating against CO, reducing its relative binding constant compared to free haem. Understanding the molecular basis of this discrimination has been the subject of a vast literature using chemically synthesized haem model compounds[3] and genetically engineered Mbs[4].

Secondly, when the three dimensional structure of Mb was solved long ago, it was noticed that there were no obvious holes or pathways through the protein to allow entrance and egress of O_2. How then can a ligand shuttle between the surrounding solvent and the active binding site? Protein dynamics provide the answer: the pathways con-

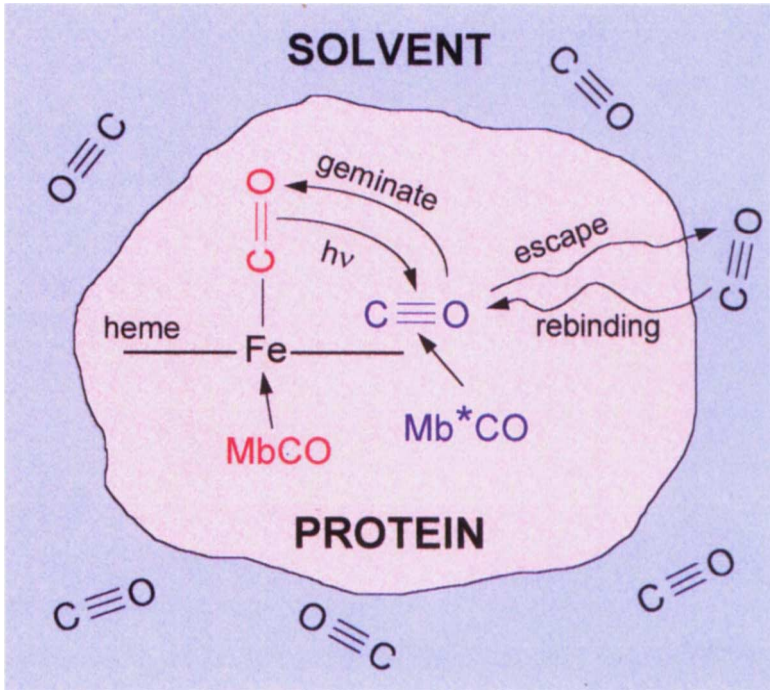

Fig 1 Schematic illustration of ligand binding and release for myoglobin. Three states are illustrated: MbCO (the CO is bound to the haem iron); Mb*CO (the geminate state structurally characterized by Teng *et al.*[1] and Schlichting *et al.*[2] where CO is photodissociated from the haem iron but remains in the protein); and Mb + CO (the fully dissociated state in which the ligand has left the protein entirely).

necting the solvent and haem are dynamic, opening and closing as the conformation of the protein fluctuates about the average structure determined by X-ray crystallography. Should we be looking for a single pathway or many pathways? It is clear now from mutational studies made in many laboratories including a random library of thousands of mutations[5], from molecular dynamics simulations[6], and from time-resolved mid-IR spectra[7] that more than one pathway exists for ligands to enter and exit the protein.

Thirdly, the three-dimensional structures of Mb and MbCO reveal subtle conformational differences. Notably, the iron in Mb is displaced approximately 0.35 Å out of the plane of the haem but is found in the plane in MbCO. The driving force for conformational change is localized, at least partially, to the iron-haem coordinate since model haem compounds with and without bound CO reveal analogous iron-haem displacements. Are such modest conformational differences relevant? The structural rearrangement accompanying conformational relaxation slows the reverse binding process and alters the free energy of binding. These effects are real and measurable and argue for adding biomechanical motion to the repertoire of control mechanisms exercised by Mb.

Spectroscopy and photolysis

How can we attain a deeper understanding of the ligand binding process? A basic problem is that ligand binding and release are thermal processes. Many years ago Quentin Gibson demonstrated that the ligand binding reaction could be studied in real time by creating the unbound state using a flash of light. As with many recent studies of caged compounds, where a flash of light can be used to create a large, non-equilibrium population of an important intermediate, photolysis of MbCO creates Mb*CO at a well defined instant; the ligand flies off in less than 100 fs. The absorption spectrum (colour) of the haem is sensitive to whether or not a ligand is bound so the reverse reaction, the rebinding of a ligand, can be studied using time-resolved spectroscopic methods.

The rebinding kinetics are more complex than expected and can be roughly divided into two phases: a short-lived phase independent of the CO concentration corresponds to Mb*CO→ MbCO and is called geminate rebinding, and a slower phase which depends on the CO concentration and corresponds to bimolecular rebinding. This slower, more complex process involves ligand diffusion through entrance channels that open temporarily due to dynamic fluctuations of the protein structure. These rebinding processes can occur over a very wide range in time and with greatly varying amplitudes dependent on the ligand, temperature and viscosity of the reaction media. For example, geminate rebinding of CO is almost non-existent at physiological temperatures, but becomes dominant at low temperature, which is one of the reasons why Teng *et al.*[1] and Schlichting *et al.*[2] performed their X-ray analyses at low temperature. For nitric oxide, a molecule long thought to be irrelevant in biology but now known to be of great importance in many processes, geminate rebinding dominates at all temperatures and is extremely fast. The physiological ligand oxygen is in between these limits: at room temperature a substantial fraction of the rebinding occurs geminately, the remainder occurring by the bimolecular pathway. Clearly the geminate pair is relevant to our understanding of the overall ligand binding process. Although much was known from X-ray crystallography about the structures of MbCO and Mb, there was no structural information on this important geminate pair until these papers.

The description of the ligand binding process in Mb, especially that of CO, has taken on a broader significance, due in part to the extensive studies of Hans Frauenfelder over the past 20 years[8]. Frauenfelder observed that the geminate rebinding process at low temperatures exhibits highly non-exponential kinetics. Because the geminate process is a unimolecular reaction, conventional kinetic treatments would predict simple exponential rebinding. Non-exponential kinetics are well known in other areas of polymer physics, especially in studies of the dynamics of glasses. Frauenfelder postulated the existence of conformational substates in proteins to explain the non-exponential rebinding kinetics. The idea is that proteins, even in their folded state, have many residual degrees of freedom so the structure of each individual protein molecule is slightly different from its chemically identical companions. These small variations in structure lead to variations in function, and the observed binding kinetics reflect this distribution.

In a significant sense, the beautiful three-dimensional structures displayed on the covers of journals like this (and on the annual reports of biotechnology companies) are deceptive: they hide some of the functionally relevant complexity associated with proteins. Of course, 'structure' means different things to different people. The positions of the atoms determined by the X-ray crystallographer reflect their time-averaged position within the unit cell over the period required to make the measurement, and, like all measurements, are subject to some degree of experimental uncertainty. Some dynamic information can be inferred from the crystallographic *B*-factors; however, it is far from clear how the *B*-factors translate into real pathways for ligand motion. To characterize an intermediate structurally, there are two choices: trap the intermediate in its pure form for the duration of the data collection, or develop time-resolved structural methods. Teng *et al.*[1] and Schlichting *et al.*[2] have chosen the first approach to obtain the first X-ray structures of Mb*CO. Due to the availability of increasingly bright X-ray sources, the time required for data collection is constantly getting shorter and shorter, however, it will be some time before one will be able to acquire a high resolution snapshot of a short-lived reaction intermediate such as Mb*CO at physiologically relevant temperatures. This is where a marriage between spectroscopy and crystallography can be extremely powerful.

Correlation with spectroscopy

Traditionally, transient absorption spectroscopy has focused on colour changes in the haem, the dynamics of which are related to the transition between the MbCO and Mb conformations characterized by X-ray crystallography. Much more detailed information can be obtained, in principle, from time-resolved vibrational spectroscopy, in which the nature and orientation of individual chemical bonds can be probed. The structure of Mb*CO provides a perfect illustration. The X-ray structure of Mb*CO was obtained at low temperature for the reasons outlined above, however, the time-resolved vibrational spectrum of CO can be measured at any temperature and with femtosecond time resolution. Anfinrud's group has shown that the mid-IR spectrum of Mb*CO at 283 K is virtually the same as that in a glass at 5.5 K[7]. The subtle differences detected can be rationalized in terms of conformational relaxation, which occurs under ambient conditions but not at cryogenic temperatures. Furthermore, because the spectrum of Mb*CO appears within 0.5 ps, the dissociated CO must be located near the haem iron, and polarized mid-IR spectra of the unbound CO show that its orientation must be parallel to the plane of the haem. This is exactly what is found in the X-ray structure of Mb*CO at cryogenic temperatures, thus the structures determined at these low temperatures really are physiologically relevant. Just as crystallography has its limitations, so does spectroscopy. While polarization spectroscopy can determine the orientation of the CO relative to the plane of the haem, it cannot specify its position in the protein. In principle, the position could be probed by measuring the mid-IR spectrum of CO photodissociated from many mutants of Mb, however, that would provide only indirect information and would therefore be subject to interpretation. Here, the X-ray structure shows quite clearly where the dissociated CO is trapped.

The results reported in these papers represent a significant and meaningful breakthrough that will surely catalyze further progress in our understanding of this reversible ligand-binding process. They also provide a wonderful example of the power of combining structural and dynamic probes of the same process to obtain a molecular view of the impact of protein structure and dynamics on function.

1. Teng, T.-Y., Srajer, V. & Moffat, K. *Nature struct. Biol.* **1**, 701–705 (1994).
2. Schlichting, I., Berendzen, J., Phillips, G.N. Jr & Sweet, R.M. *Nature*, **in the press** (1994).
3. Collman, J. P., Brauman, J.I. & Doxsee, K.M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6035–6039 (1979).
4. Springer, B.A., Sligar, S.G., Olson, J.S. & Phillips, G.N. Jr *Chem. Rev.* **94**, 699–714 (1994).
5. Huang, X. & Boxer, S.G. *Nature struct. Biol.* **1**, 226–229 (1994).
6. Elber, R. & Karplus, M. *J.Am.chem.Soc.* **112**, 9161–9175 (1990).
7. Anfinrud, P.A., Lim, M. & Jackson, T.A. (manuscripts in preparation).
8. Frauenfelder, H., Sligar, S.G. & Wolynes, P.G. *Science* **254**, 1598–1603 (1991).