REVIEW

Myoglobin strikes back

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Abstract: Over the last half century, myoglobin (Mb) has been an excellent model system to test a number of concepts, theories, and new experimental methods that proved valuable to investigate protein structure, function, evolution, and dynamics. Mb's function, most often considered just an oxygen repository, has considerably diversified over the last 15 years, especially because it was shown to have a role in the biochemistry of quenching and synthesizing nitric oxide in the red muscle, thereby protecting the cell. To tackle protein's structural dynamics by innovative biophysical methods, Mb has been the best prototype; laser flash technology made it possible to obtain molecular movies by time-resolved Laue crystallography (with ps resolution). This approach unveiled the complexity of the energy landscape and the structural basis of the stretched interconversion between conformational substates of a protein.

Keywords: myoglobin; H-atom; molecular biology; protein science

Introduction

The three-dimensional structure of myoglobin (Mb), the hydrogen atom of molecular biology, was solved 50 years ago by Kendrew *et al*. A preliminary report of the structure appeared in Nature in 1958[1]; a more detailed account was published the following year in the Proceedings of the Royal Society[2]; and the full paper in Nature on 13 February 1960.[3]

For half a century, Mb has been a popular model system to test a number of concepts, theories, and new experimental methods dealing with protein structure, function, dynamics, and evolution. Hereby, I summarize the novelties in the physiological role of Mb in the heart and the red muscle over-and-above O_2 storage and outline some of the experimental and conceptual advances on protein's structural dynamics that emerged from time-resolved Laue crystallography on Mb, with ps time resolution. This short review, a tribute to Sir John C. Kendrew and coworkers, is being published just 50 years since the Nature paper thanks to the collaboration of the Editor of Protein Science.

Just an Oxygen Repository?

Our understanding of the functional role of Mb expanded considerably over the last 15 years, because it became clear that the protein is involved in controlling the fluxes of nitric oxide (NO) in the heart, under physiological and pathological conditions. Generally, Mb's function in most peoples' mind, and indeed even in Biochemistry text books, is limited to its role as an O_2 storage, a minimalist misnomer. This depends largely on the (correct) notion that in diving mammals, whose muscles look black because the concentration of Mb is ~10-fold greater than in humans, its role as an O_2 repository is physiologically important; in man and the other mammals, it was calculated[4] to be sufficient to

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sustain aerobic metabolism for approximately a couple of seconds, that is, for O_2 supply in between beats.

Probably, more significant but generally ignored is the role of Mb in facilitating O_2 diffusion from capillaries to mitochondria, given its high concentration (100–300 μM) and free translational diffusion in the intracellular milieu.[5,6] Interestingly, although a transgenic Mb knockout mice ($myo^{-/-}$) were reported to behave normally,[7] histology of a section of the heart showed that the density of capillaries in the knockouts was increased,[8] one of the compensatory responses to reduce the effective diffusion path length. This was a striking unexpected evidence that indeed Mb plays a significant role in facilitating O_2 diffusion to mitochondria, thereby keeping the sarcoplasmic O_2 pressure essentially constant throughout the cell and Mb approximately half-saturated with O_2, independently of the workload.[6]

More recently, NO moved Mb center stage, once it was discovered in 1994 that cytochrome c oxidase, the terminal enzyme of the respiratory chain, is reversibly inhibited by NO.[9,10] Even at low physiological fluxes (<0.1 μM), NO is a potent poison of respiration; the apparent inhibition constant depends on O_2 concentration because the two gases compete for the oxidase active site, which may explain[11] why the apparent K_m for O_2 in cells and tissues (5–10 μM) is significantly greater than that measured with mitochondria or the purified enzyme ($K_m < 1$ μM). The mechanism of inhibition of cytochrome c oxidase by NO, which is not simple, has been largely clarified by extensive presteady-state and steady-state experiments.[12–14] In 1998, Moncada's group[15] discovered that also Complex I is inhibited by NO albeit via a different biochemical reaction, which explains why speed of inactivation and recovery in the latter case are both much slower than for the case of oxidase.

Starting from these premises it seemed obvious to me[16] that one important and hitherto overlooked function of Mb in the skeletal and cardiac muscle may be scavenging NO, thereby protecting the energy-producing machinery. The mechanism of quenching is simple and effective [Eq. (1a)], because oxygenated MbO_2 reacts rapidly and stoichiometrically with NO, yielding nitrate and ferric Mb^{+}[17]; the presence of an intracellular Mb^{+} reductase ensures rereduction to ferrous Mb and thereby a catalytic cycle.

$$MbO_2 + NO \rightarrow Mb^{+} + NO_3^{-} \quad (1a)$$

$$Mb + NO_2^{-} + H^{+} \rightarrow Mb^{+} + NO + OH^{-} \quad (1b)$$

Flögel *et al.*[18] published the first convincing evidence in support of this hypothesis. These authors found that typical cardiac physiological parameters (e.g., coronary blood flow and cardiac output) were more severely affected by NO fluxes in the heart of the knockout mice lacking Mb ($myo^{-/-}$) compared with the wild type. The fact that the same NO flux has greater adverse effects in the hearts from $myo^{-/-}$ mice may be understood assuming a more substantial inhibition of cytochrome c oxidase by NO in the knockouts.

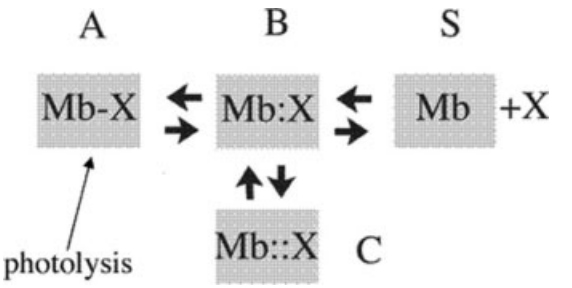

Figure 1. Simplified kinetic scheme depicting events following photolysis of liganded Mb (A). The photolysed ligand (O_2, CO, NO, and others) populates the so-called distal pocket (primary docking site, B), and thereafter may: (i) diffuse out into the solvent (S) via the His-gate,[26] the most probable path according to Scott *et al.*[27]; (ii) rebind to the metal by an intramolecular geminate process B → A; (iii) diffuse into the matrix secondary docking sites (C), following a path traced by the Xe binding cavities (as shown by time-resolved Laue crystallography). Escape out into the solvent is occurring also via other pathways over-and-above the His-gate.[28,29]

Over the last few years, yet another function of Mb related to NO metabolism in the heart has been substantiated.[19,20] It was shown both *in vitro* and in the heart[21] that under anaerobic conditions deoxy Mb is endowed with nitrite reductase activity [see Eq. (1b)], thus producing NO just when NO synthase is inactive because of the lack of O_2. The rate of NO synthesis from NO_2^{-} (at ~10 μM in tissues[21]) depends on deoxy Mb, because it becomes undetectable in the $myo^{-/-}$ knockouts. Under anaerobiosis, Complex I and Complex IV of the respiratory chain are both inhibited by NO[9,10,15]; thus, reoxygenation is not associated with the classical ischemia-reperfusion ROS production and tissue damage is reduced. The cardioprotective effect is so evident[22,23] that clinical trials to assess the positive effect of nitrite administration on the extent of heart damage are under course.

A Paradigm of Complexity

Mb has been an ideal model system for experiments on the structural dynamics of proteins and its role in controlling function, because it is sufficiently simple to be investigated in detail but complex enough to be intriguing and nontrivial. An extremely useful property of Mb is the photosensitivity of the ligand adduct, the complex of ferrous Mb with O_2, CO, NO, and other ligands being light sensitive[24,25]; thereby hitting liganded Mb with a short and intense laser

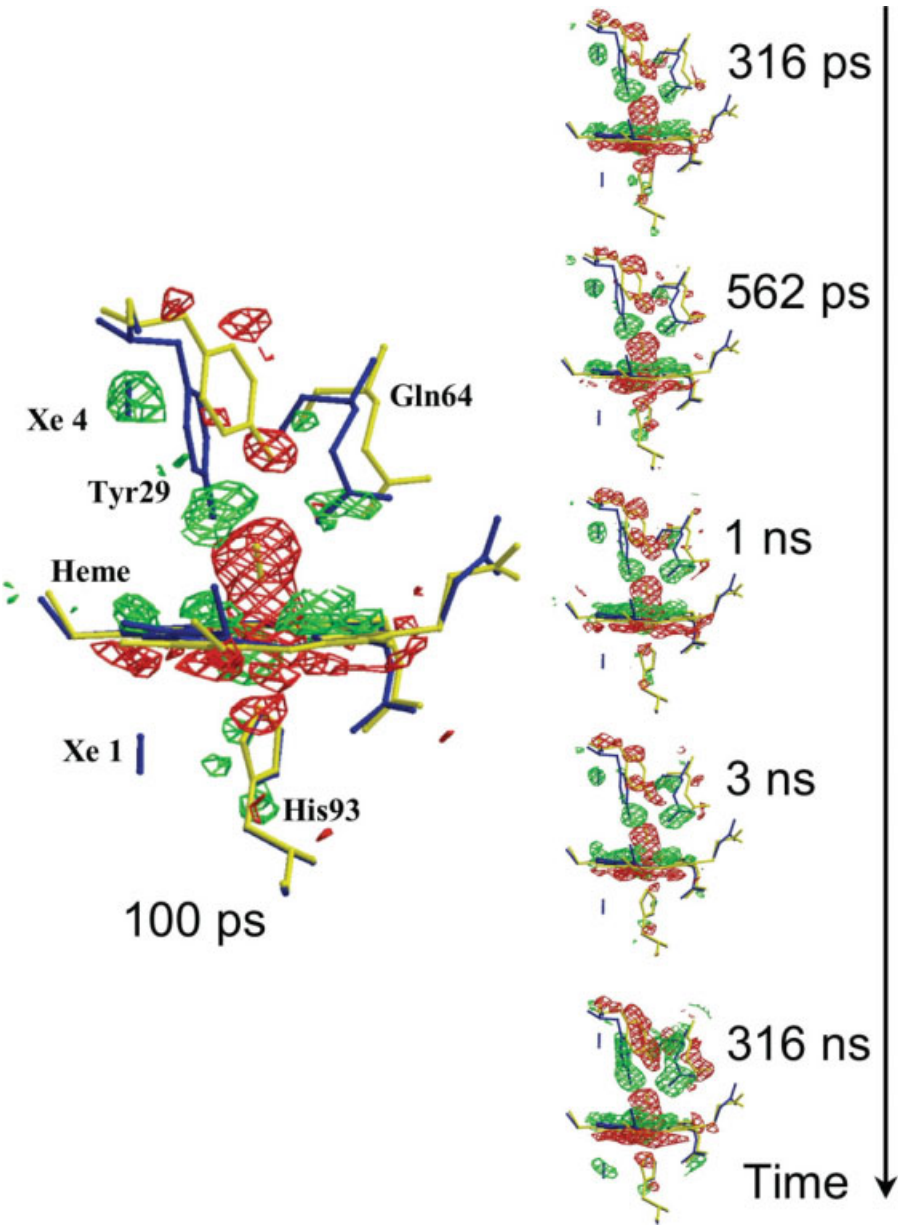

Figure 2. Structural changes in the heme vicinity for the mutant called YQR-Mb,[43] from 100 ps to 316 ns after CO photolysis. YQR-Mb is a triple mutant (L29Y/H64Q/T67R) used for this experimental session because of favorable properties (zero CO geminate recombination, slow bimolecular rebinding, and excellent sturdy crystals). ($F_{light} - F_{dark}$), the difference electron density maps, are in red for negative and in green for positive (contoured at 3.0 σ); these are overlaid on models of YQR-MbCO (in yellow) and YQR-Mb (in blue). It may be seen that at 100 ps, the negative density (red) of the $Fe^{2+}CO$ on the distal side of the heme is very prominent; other detectable changes are the initial distortion of the heme and motions of the distal Tyr29 and Gln64. These structural changes continue to grow with time and are fully developed in the frame at 316 ns. In all the frames, a blue bar indicates the position of photolyzed CO migrating initially to the Xe4 pocket (distal, at the top in the 100 ps frame) and later to the Xe1 pocket (proximal, at the bottom, better seen in the frame at 316 ns) (from Bourgeois *et al.*,[44] modified).

pulse, breaks the ligand-iron bond and sets into motion the relaxation of the photoproduct, deoxy Mb. In the late 70s, this approach led to the discovery of the so-called geminate recombination, that is, the intramolecular rebinding to the iron of the photodissociated ligand trapped momentarily within the protein matrix (Fig. 1). Although the structural differences between the two equilibrium states (deoxy and liganded Mb) are not large, kinetics showed their functional importance. The kinetics of Mb has been extensively investigated by transient laser spectroscopy and sophisticated modeling by many investigators; among others, pioneering contributions were reported by Q.H. Gibson, H. Frauenfelder, W.A. Eaton, and their groups.[27,28,30–34] Below is a summary of the main features of the relaxation kinetics of the whole protein as revealed by time-resolved Laue crystallography, a global approach.

The classical trick to obtain structural information on short-lived states is to freeze intermediates at ultra-low temperatures (e.g., ~10° K). This approach applied to the photoproduct of MbCO, allowed Schlichting *et al*.[35] to prove that photodissociated CO is indeed located in the so-called distal pocket. Several other papers[36–39] exploiting the low-temperature trapping method confirmed and extended this initial finding and substantiated, for example, the migration of CO inside the protein upon increase in the temperature of the crystal.[36,37] Nevertheless, it was obvious that direct information to unveil the structural relaxation of the globin was to come from room temperature time-resolved crystallographic data.

This difficult task has been for 2 decades the *mission impossible* of Keith Moffat and his collaborators, who published in 1996[40] the first reliable time-resolved Laue diffraction experiment; the quality of those results bears no comparison with current data, but it was nevertheless an important breakthrough showing that the experiment was possible and informative. In the following years, substantial improvements in synchrotron technology, data analysis, and protein biochemistry[41,42] made it possible to carry out a high-resolution structural characterization of Mb's dynamics from 100 ps after photolysis up to many ms, covering the huge time domain typical of protein relaxations.

Intrinsic difficulties of photolysis in single crystals are (i) the limited time span available to shoot X-ray pulses before CO bimolecular recombination in the dark, and (ii) the low yield of deoxy Mb photoproduct due to geminate rebinding. Site-directed mutagenesis proved valuable to minimize these shortcomings; for example, the triple mutant called YQR-Mb (L29Y/H64Q/T69R[43]) was a convenient variant because the mutations in the distal pocket abolish CO geminate recombination and slow down (by 10-fold) bimolecular rebinding, extending the time window accessible to interrogation with short (150 ps) X-ray pulses.

The new generation of Laue diffraction experiments led to a complete characterization of the global structural relaxation of Mb after CO photodissociation (Fig. 2). Wild-type sperm whale Mb and three mutants were investigated in detail.[44–48] Data obtained by the different groups were by-and-large consistent and allowed to unveil a few features of general significance for the structural dynamics of

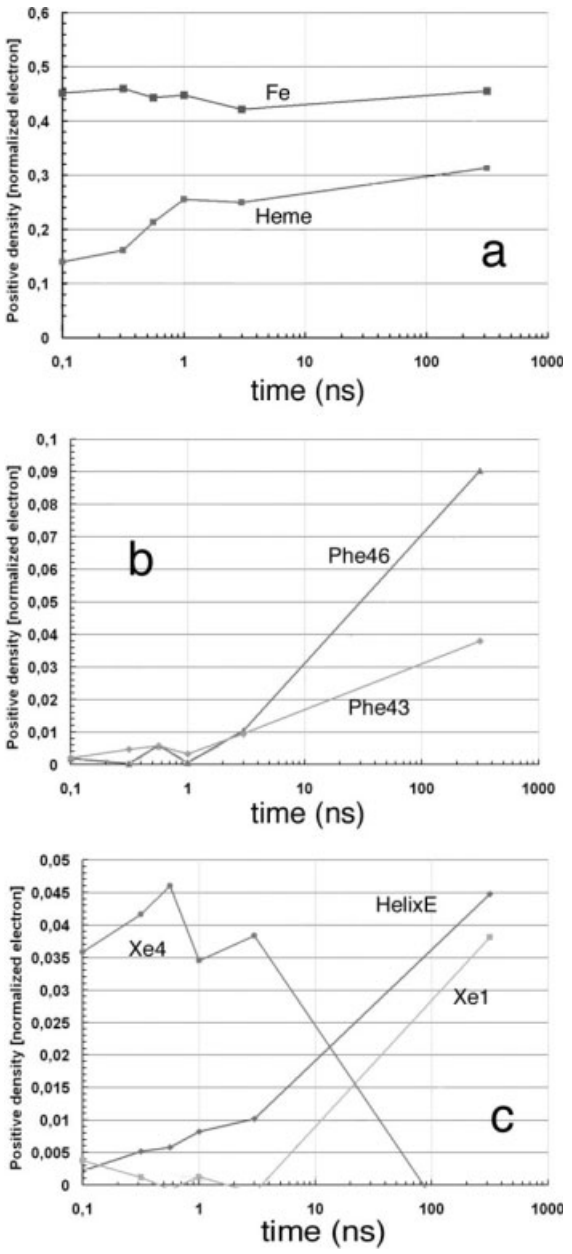

Figure 3. Time dependence of difference electron densities for key structural features, after photolysis of YQR-MbCO. Numerical values represent the integral of the positive electron density beyond 3.0 σ, corrected for variations in photolysis yield and normalized to the negative bound CO feature (arbitrary value of 1). (a) Key features that appear promptly: the density of the Fe popping out of the heme plane without delay remains time independent; while distortion of the heme (see Fig. 2) and motion of Tyr29 follow an heterogeneous time course being already detectable in the first frame (density of a 0.10–0.15 at 0.1 ns) but increasing with time. (b) Amino acid residues involved in the strain of the CD turn clearly lag behind the conformational changes of the heme and of Tyr29 on the distal side (see above). (c) Population of CO in the Xe1 and Xe4 pockets, and conformational changes of the E-helix: CO appears promptly after photolysis in the Xe4 pocket, on the distal side of the heme (with 0.035–0.045 density), and begins to diffuse inside and populate the Xe1 pocket on the proximal side starting from ~5 ns. The migration of CO is approximately synchronous with larger scale conformational changes of the E-helix, suggesting that long-scale intramolecular diffusion demands more substantial structural changes of the globin. At much longer times (e.g., toward ms) CO rebinds largely via the opening of the His-gate,[26] as shown by Scott *et al*.[27] studying many different mutants of sperm whale Mb (from Bourgeois *et al*.,[44] modified).

proteins at large. In all cases, the structural relaxation of the deoxy Mb photoproduct was found to be nonexponential extending from ps to μs and above, in agreement with spectroscopic data.[49] Subtle differences in dynamic behavior between the mutants and the wild type were largely rationalized and correlated with transient optical spectroscopy in solution.

The relaxation profile of the heme and of the globin moiety (Fig. 3) extends over a time range from 100 ps (the earliest available frame) to μs and above, until CO rebinding in the dark leads back to the equilibrium state.[45] The heme relaxation is heterogeneous, some of the tilt being synchronous with the laser pulse and the rest occurring within several ns; in YQR-Mb heterogeneity was correlated[44] to a strain on heme pyrrole C exerted by the E-helix via the CD turn. The larger conformational changes of the globin develop with a lag of ~50–100 ns, as may be seen by looking at the relaxation of the distal E-helix and several residues of the CD turn [Fig. 3(b,c)]. The stretched relaxation behavior is consistent with the idea of a *protein quake* (championed by Frauenfelder and coworkers[50]), whereby photolysis sets into motion faster changes in the immediate environment of the heme, and larger but slower perturbations with rearrangements of the globin involving conformational changes of the helices. This complex relaxation behavior was taken to represent interconversion between sets of conformational substates jumping over multiple barriers, with or without solvent displacement.[51]

Associated with these extended conformational changes, migration of the photolyzed ligand inside the protein was observed directly.[44–48] CO diffusing from the so-called distal site near the heme to a cavity located just above the distal pocket, the so-called Xe4 (Fig. 2, at 100 ps), and subsequently to another prominent cavity located on the proximal side (so-called Xe1) (Fig. 2, at 316 ns). The electron density of CO while migrating between cavities and its time dependance [Fig. 3(c)] were by-and-large the same for all Mb variants; the recovery of CO was carefully analyzed by Srajer *et al*.[45] and compared to the recombination time course. The crystallographic time-resolved data are in satisfactory agreement with geminate recombination studies on different ligands (CO, NO, and O_2) obtained in solution, and particularly with the effects of mutations and Xe binding on the kinetics.[27,52]

These extended data sets led to some general conclusions. The stretched globin relaxation is a direct and original demonstration of the complexity of the energy landscape of a protein.[53] This envisaged a multiplicity of paths as the protein skates along a complex set of conformational coordinates, indicated by the complex kinetics and the effects of mutations, temperature, and viscosity.[27,34] A fundamental point yet under debate is whether the stretched structural changes (Fig. 3) are an intrinsic property of each individual molecule relaxing in a smooth extended process or an average behavior of all molecules[54]; possibly single molecule kinetics may solve this point.

The reliable structural information now available from a few hundred ps allowed a quantitative comparison between experimental data and molecular dynamics simulations. It was rewarding to find out that experiments and simulations[55,56] are in good agreement, a result of great value for the future of structural dynamics. Simulations may allow a clearer identification of essential dynamic features that may have escaped examination of the ensemble averaged difference maps; moreover, the necessarily limited number of experimental points along the extended time course can be filled by the simulations. Finally, mutual validation paves the way to wider applications of MD to investigate the stretched conformational relaxation of proteins that are not amenable to laser-triggered Laue diffraction experiments.

The ambition of the structural dynamics approach was to prove in selected cases that direct determination of global conformational changes related to function is feasible, with remarkable time and spatial resolution. The experimental evidence of a stretched relaxation is one of the most convincing proofs of the complexity of the protein energy landscape and the existence of functionally relevant quasi isoenergetic conformational substates. More work is foreseen, such as the acquisition of tertiary relaxation data for the R and T states of hemoglobin, following the path opened by the pioneering studies of Keith Moffat and others exploiting time-resolved Laue crystallography. Based on half a century of honorable service, it seems impossible to ignore the role of Mb as the paradigm of complexity in protein structural dynamics.

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