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Journal of Structural Biology 147 (2004) 223–234
Journal of Structural Biology
www.elsevier.com/locate/yjsbi

The structural dynamics of myoglobin☆

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Received 23 December 2003, and in revised form 15 March 2004
Available online 19 May 2004

Abstract

Conformational fluctuations in proteins were initially invoked to explain the observation that diffusion of small ligands through the matrix is a global phenomenon. Small globular proteins contain internal cavities that play a role not only in matrix dynamics but also in controlling function, tracing a pathway for the diffusion of the ligand to and from the active site. This is the main point addressed in this Review, which presents pertinent information obtained on myoglobin (Mb). Mb, a simple globular heme protein which binds reversibly oxygen and other ligands. The bond between the heme Fe(II) and gaseous ligands can be photodissociated by a laser pulse, generating a non-equilibrium population of protein structures that relaxes on a picosecond to millisecond time range. This process is associated with migration of the ligand to internal cavities of the protein, which are known to bind xenon. Some of the results obtained by laser photolysis, molecular dynamics simulations, and X-ray diffraction of intermediate states of wild-type and mutant myoglobins are summarized. The extended relaxation of the globin moiety directly observed by Laue crystallography reflects re-equilibration among conformational substates known to play an essential role in controlling protein function.

Keywords: Protein crystallography; Laue crystallography; Photolyis; Intermediate states; Structure–function relationships; Myoglobin; Cavities

1. Protein conformation and control of function

It is widely accepted that the biological function of many proteins is controlled by conformational changes of different magnitudes. These crucial structural changes are seldom characterized at atomic level resolution and even less is known about their dynamics. Often the 3D structure of the initial state of an enzyme and its complex with an inhibitor or a substrate analogue are available. However, this information, albeit crucial for the molecular interpretation of biochemical data relevant to the reaction mechanism, yields no clue to the dynamics of the conformational transitions of the whole protein "in action."

If proteins were rigid objects they would be essentially dead. The crystallographic structure of a protein yields high resolution information on the time-average state of the macromolecule, but a static view point may be misleading (Frauenfelder et al., 1988). In fact, a huge amount of experimental data shows that ligand and substrate binding, partner recognition, control of transport, and catalysis (and in general regulation of activity) demand flexibility. Of course, relevant information on protein's dynamics in solution can be obtained by spectroscopic techniques such as NMR, X-ray absorption spectroscopy, fluorescence resonance energy transfer, and others. In this article, however, we shall limit ourselves to summarize progress on Mb structural dynamics as obtained by X-ray crystallography, which provides a view of the whole protein, often at quasi-atomic resolution. In the past, dynamic information has been obtained from the temperature dependence of the B-factors or rms deviations of atomic positions in a crystallographic structure; such an approach was applied, for example, to ferric Mb by Frauenfelder et al. (1979) in an attempt to provide a structural interpretation of the complex CO rebinding kinetics observed at

☆ Supplementary data associated with this article can be found in the online version, at doi: 10.1016/j.jsb.2004.04.008.

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doi:10.1016/j.jsb.2004.04.008

low temperatures (Austin et al., 1975). Nowadays direct and reliable structural information by time-resolved Laue crystallography is available (Bourgeois et al., 2003; Schotte et al., 2003; Srajer et al., 2001).

Flexibility as a concept is not easy to correlate with structure and overall stability. Some understanding of the correlations between function, flexibility, and overall thermodynamic stability has been achieved by comparing, for example, the properties of enzymes from mesophilic and thermophilic micro-organisms. It is known that the folded state of a protein corresponds to a free-energy minimum that is sufficiently deep to maintain the native structure populated under physiological conditions, but often marginally above a critical value; after all, the thermodynamic stability of many globular proteins is around (or just above) 10 kcal mol^{-1} (the free-energy level of a couple of H-bonds) (Creighton, 1991; Fersht, 1999). By studying proteins and enzymes from thermophilic micro-organisms it was shown that their increased stability is invariably associated with restrained dynamics and an essentially inactive state at room temperature (see Jaenicke, 1999 and references therein).

Understanding the general rules which govern the prevailing structure of a protein and defining a theoretical framework to describe its dynamic behaviour are, in our opinion, essential goals for the structural biology of our time. Time-resolved spectroscopy in solution has shown that the dynamic range of protein conformational changes is very wide, extending from picosecond to millisecond and above. Thus, it may be stated that the next frontier of structural biology is the elucidation of the structure of a protein at atomic resolution, over the whole time range of relevance to biology. Time-resolved Laue crystallography has proven to be a powerful approach, but we are just at the beginning of this era. Here we present our viewpoint on the structural dynamics of Mb, the paradigm of complexity (Frauenfelder et al., 2003) being sufficiently simple to allow a rigorous definition of the important questions, but complex enough to serve as a model system of general significance.

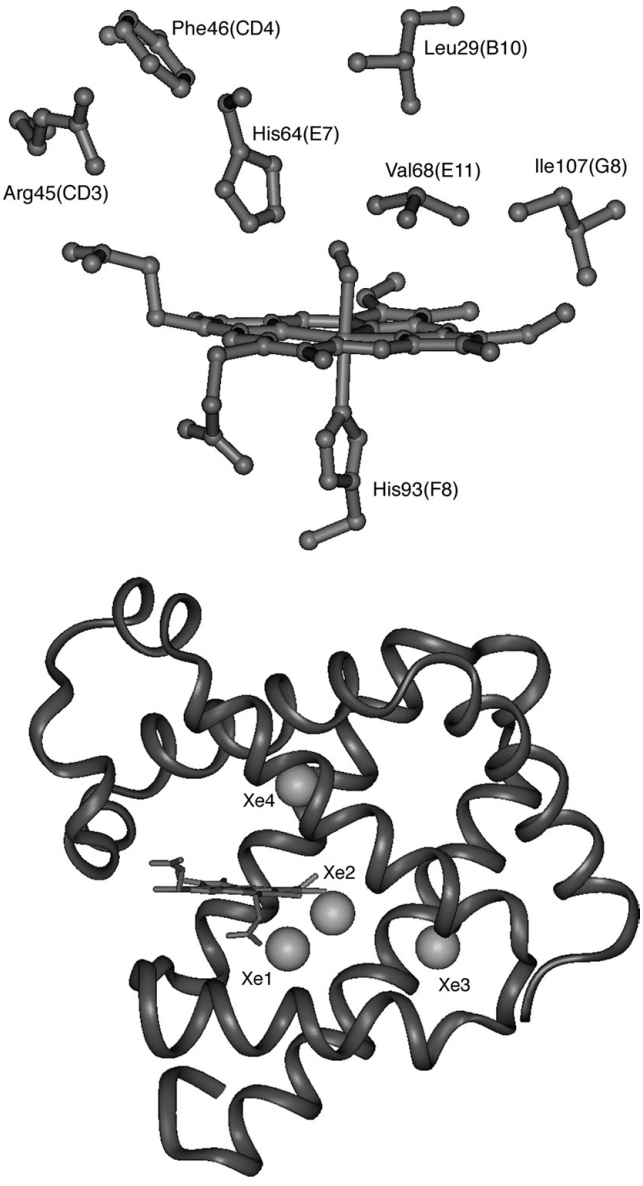

Fig. 1. (Top panel) The active site of sperm whale oxy Mb: some amino acid side chains in the immediate environment of the heme are indicated. (Bottom panel) Overall structure of sperm whale Mb (with the heme in the same orientation as above), displaying the location of the four xenon atoms bound in matrix cavities, shown as spheres.

2. Why myoglobin?

Mb is primarily (but not exclusively) involved in the delivery of oxygen to the mitochondria in the red muscle. Oxygen is bound to the ferrous heme iron with an overall affinity intermediate between that of mitochondrial cytochrome-*c*-oxidase (the terminal enzyme of the respiratory chain) and that of hemoglobin, the oxygen carrier in the blood (Antonini and Brunori, 1971). The deoxygenated state of Mb binds many other ligands, including CO and NO which display higher affinity than O_2. A picture of the active site of sperm whale Mb, shown in Fig. 1, highlights a number of amino acid side chains surrounding the heme, which were shown to play a primary role in controlling the binding of gaseous and non-gaseous ligands, and in the discrimination among them (Springer et al., 1994).

Mb has enjoyed a central position as a model system to investigate protein dynamics and its role in controlling binding and dissociation of ligands. This pivotal role stems from the availability of several ligands of ferrous Mb characterized by different intrinsic reactivities (Antonini and Brunori, 1971; Wittenberg, 1970), and from the photosensitivity of the iron-ligand bond (Ainsworth and Gibson, 1957) whereby illumination of the adduct of ferrous heme with a ligand leads to

rupture of the coordination bond. Moreover the large number of site-directed mutants available, and the variety of proteins belonging to the *family* as defined by the classical *globin fold* has extended considerably the appeal of hemeproteins for structural dynamics investigations. Suffice here to recall that over the last years, a number of new globins with different molecular weights and active site structures, providing interesting *variations on the theme*, has been discovered and characterized (Bolognesi et al., 1997; Burmester et al., 2000; Hargrove et al., 2000; Pesce et al., 2002; Wittenberg et al., 2002).

Examination of the model of Mb (Kendrew et al., 1960) immediately indicated that there was no obvious channel for the diffusion of O_2 to the heme iron; thus, some mobility of the protein was presumed to be essential for the diffusion of the ligand to the active site. An initial hypothesis based on the structure of azide-met hemoglobin (Perutz and Mathews, 1966) suggested that a channel may be opened by a swinging motion of the distal His(E7)64, which may rotate relative to its crystallographic position. This hypothesis has been essentially substantiated, initially with work on Mbs from different species (e.g., Bolognesi et al., 1982) and subsequently by very extensive and quantitative experiments on many site-directed mutants (Olson et al., 1988; Scott et al., 2001). Moreover pioneering molecular dynamic simulations have provided a picture of the motions of the active site residues and identified possible escape channels for the ligand to reach the bulk (Elber and Karplus, 1990; Kuczera et al., 1993).

3. Multiplicity of states: the energy landscape

Over the last 20 years, laser spectroscopy with time resolution as fast as femtosecond (Petrich et al., 1988) has provided a wealth of novel information. A conceptual breakthrough originated from the discovery of the so-called *geminate state*, and the introduction of the concept of conformational substates (Austin et al., 1975; Frauenfelder et al., 1988). The pioneering experiment by Austin et al. (1975) opened a novel perspective on protein dynamics and reactivity, and paved the way to the concept of the *energy landscape*. Photodissociation of MbCO below 150 K showed that ligand rebinding is highly non-exponential in time, and is characterized by a peculiar and unexpected temperature dependence. To account for these observations, it was proposed that Mb populates a large number of conformational substates, which are frozen at cryogenic temperatures and which express, under those conditions, large differences in rebinding rate (Ansari et al., 1985; Frauenfelder et al., 1988). A first attempt to correlate this view with the 3D structure of the protein made use of the determination of the temperature dependence of B-factors in crystals of sperm whale met-Mb (Frauenfelder et al., 1979).

Ever since, it is assumed that Mb (but possibly every protein) has a complex energy landscape (Frauenfelder et al., 1991), the modern conceptual framework to describe the motions of a macromolecule and the dynamic control of reactivity. The energy landscape is a representation of the protein whereby the conformational substates are organized in a hierarchical set and supposedly interconvert at room temperature in a complex array; accordingly, the number of possible pathways between substates is large and may involve complex transitions. This view of the protein conformational landscape paved the way to the conception of the funnel theory to describe protein folding (Wolynes et al., 1995), the most extreme of all conformational changes. In summary, the native protein does not assume a unique structure, but populates a large number of somewhat different conformational variants. Careful studies carried out by time-resolved laser spectroscopy at room temperature as a function of solvent viscosity showed that ligand binding is coupled to the internal friction of the protein matrix (Ansari et al., 1994). The dynamics of the conformational changes of the whole globin, recently unveiled at room temperature by time-resolved X-ray diffraction experiments, is a direct evidence for transition between substates, as detailed below.

4. Mutants, intermediates, and mechanisms

Time-resolved laser spectroscopy at room temperature showed that early recombination of a photolyzed ligand occurs from within the protein matrix, the so-called *geminate recombination* (Duddel et al., 1980; Gibson, 1989; Henry et al., 1983). Rebinding from a geminate state (Fig. 2) is ligand concentration independent and thus can be easily discriminated from bimolecular rebinding involving diffusion of the ligand from the bulk to the active site (Antonini and Brunori, 1971). After photodissociation, the diatomic molecules (initially bound to the ferrous iron) initiate a rapid migration away from the heme, and thereby probe the accessibility of the protein interior. It was soon realized that the geminate intermediate obtained by photodissociation is not a single species, and in fact it was proposed that multiple geminate states are populated (see scheme in Fig. 2). Using mutagenesis, the role of specific residues coating the active site was shown to control geminate rebinding (see for review Scott et al., 2001 and ref. therein). These studies extended the observations of Austin et al. (1975), and paved the way to a series of extremely important investigations aimed at the interpretation of the multiplicity of geminate states, which were attributed to a variable inner barrier and/or to migration of the photolyzed ligand to secondary

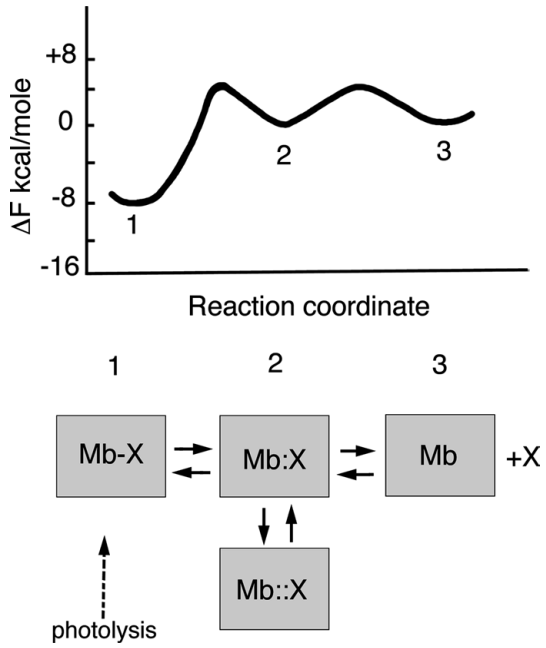

Fig. 2. Energy diagram of a 3-states landscape and branched scheme for ligand binding and dissociation to Mb.

docking sites. Reviewing even the pivotal contributions in this field is outside the scope of this article, but a few among the many excellent papers may be quoted for the interested reader (Agmon and Hopfield, 1983; Ahmed et al., 1991; Ansari et al., 1994; Nienhaus et al., 1992; Steinbach et al., 1991).

Unequivocal evidence for geminate rebinding after photolysis of MbCO at room temperature was initially very difficult to acquire (Henry et al., 1983). However, improvements in technology, variations in solvent conditions, and the use of different ligands (especially NO and O_2) have subsequently clarified the picture (see Gibson, 1989; Olson and Phillips, 1997 for reviews). The significance of site-directed mutants of Mb, first produced by Varadarajan et al. (1985) and by Springer and Sligar (1987), cannot be overemphasized. Among the outstanding results obtained by this approach was the proof that the H-bond between the distal His(E7)64 and bound O_2 is essential for the stability of the adduct (Olson et al., 1988), as reviewed by Perutz (1989). Mutation of His(E7)64 to a hydrophic residue increases the O_2 dissociation rate constant from $\sim 10\,s^{-1}$ typical of wild type Mb (Antonini and Brunori, 1971) to $\sim 10\,000\,s^{-1}$, with a dramatic effect on affinity and stability towards autoxidation. Some natural Mbs lacking His at position (E7) are nevertheless capable of forming a stable oxygenated state; in these cases other distal residues which stabilize the O_2 complex are present, such as Gln(E7) in elephant Mb (Bartnicki et al., 1983) and Arg(E11) in *Aplysia l.* Mb (Bolognesi et al., 1990).

Approximately 200 different mutants of sperm whale Mb have been produced and carefully characterized, often also by crystallography (Olson and Phillips, 1997; Scott et al., 2001). By-and-large these were designed either to control the rates of ligand binding/dissociation or to probe the channels connecting the distal pocket to the matrix. The work on mutants, lucidly reviewed by Scott et al. (2001), indicates that the majority (~80%) of the photodissociated ligand escapes to the bulk via the His(E7)64 gate. Other mutants allowed to show that the mechanism of discrimination between different ligands (O_2, CO, and NO) is essentially electrostatic (Olson and Phillips, 1997), or were used to achieve a significant control of the rate of O_2 dissociation (down to approx. $0.5\,s^{-1}$) and of NO combination (down to approx. $10^6\,M^{-1}\,s^{-1}$ compared to $10^8\,M^{-1}\,s^{-1}$ for wt). The triple mutant called Mb-YQR (Leu29(B10) Tyr, His64(E7)Gln, and Thr67(E10)Arg), which we synthesized in an attempt to mimic the Hb from the worm *Ascaris suum* (Brunori et al., 1999; Travaglini-Allocatelli et al., 1994), proved to be particularly informative for structural dynamics investigations.

5. Cavities and geminate rebinding

Ever since the three-dimensional structure of lysozyme was determined, it has been observed that enzymes are often characterized by a crevice near the active site, which is shaped to host the substrate. For large enzymes, a channel guiding to the active site has been often identified; in some more complex metabolic pathways, evidence for direct channelling of a substrate from one enzyme to the next has been observed (Miles et al., 1999). However, also small globular proteins, which most often have no large channels, display a number of internal packing defects and small cavities, as reviewed by Richards (1977).

Kendrew and coworkers (Shoenborn et al., 1965) showed that Mb can bind xenon atoms when crystals are exposed to 2.5 atm or more of this gas. The binding of Xe, due to van der Waal's forces, is completely reversible and very weak. Twenty years after the first report, the structure of met-Mb equilibrated at room temperature with Xe (at 7 atm) showed the presence of four binding sites (Fig. 1) corresponding to pre-existing cavities, which are lined by predominantly hydrophobic side chains. The sites were numbered by Tilton et al. (1984) according to the relative occupancy of the xenon atoms; site Xe1 is almost fully occupied at 7 atm and is located on the proximal side of the heme pocket, just under the heme. The other three sites are partially occupied (from 40 to 30%): Xe2 is at the edge of the heme, close to Xe3, which is the closest to the external surface of the protein; Xe4 sits above the distal heme pocket. In the last 10 years, many more cases of Xe binding in internal protein cavities have been observed, with a *crescendo* of interest also due to the use of the electron-

rich Xe atoms to produce isomorphous derivatives for phase determination (Quillin and Matthews, 2002 and ref therein).

These internal packing defects ($\sim$40–80 Å^3) are known to reduce thermodynamic stability, the cost of creating a cavity of the size of methane being $\sim$5 kcal mol^{-1} (Lee, 1993). In agreement thermophilic proteins, which are more stable than their mesophilic counterparts (Jaenicke, 1999), are often devoid of these cavities (Szilagyi and Zavodszky, 2000). Why Evolution has not cured these packing defects, given that they destabilize the protein? An explanation emerging from recent studies on Mb is that cavities not only are essential components of flexibility, but define an internal pathway for the migration of the ligand to and from the active site, with a control of function (Brunori and Gibson, 2001). A convincing correlation between packing defects and protein reactivity in Mb came from an experiment whereby occupation of the cavities by Xe changed the geminate O_2 rebinding profile, as published by Scott and Gibson (1997). These authors showed that the geminate yield and the geminate rates were both affected if Mb was equilibrated with xenon at high pressures, in such a way that filling the holes with Xe refrained the ligand diffusion within the protein. Work based on mutagenesis extended our understanding of the role of cavities in geminate yield and bimolecular rates under physiological conditions (Gibson et al., 1992; Nienhaus et al., 2002).

These considerations allowed to propose that the complexity of the geminate state is related to the internal structure of the protein, not only in the immediate surroundings of the heme (the so-called distal side), but also further away within the protein matrix. Migration of the ligand in the internal Xe-binding cavities has been initially demonstrated by ultra-low temperature (20–100 K) crystallography of photolyzed MbCO, and more recently by time-resolved Laue crystallography at room temperature.

6. Structure of "frozen" intermediates

Geminate recombination implies the presence of reaction intermediates in the photoproduct state (see Fig. 2). Direct information on the structure of intermediate states emerged from pioneering crystallographic work on photolyzed MbCO at ultra-low temperatures (20 K) (see Schlichting and Chu, 2000 for review). The use of CO is justified because the photochemical yield for this ligand is almost unity (Ainsworth and Gibson, 1957; Antonini and Brunori, 1971), and thus more photolysis can be achieved. Moreover, CO can be followed by IR spectroscopy which made it possible to characterize photoproduct intermediates at different temperatures (Chu et al., 1995; Lim et al., 1993; Nienhaus et al., 1994). The initial crystallographic experiments published by Schlichting et al. (1994) and by others (Hartmann et al., 1996; Teng et al., 1994), showed that the photodissociated ligand is located near the active site, about 3–4 Å from the heme iron in the so-called distal pocket; almost no changes in the side chains surrounding the heme were seen because of the frozen motions at ultra-low temperatures. The picture became more interesting when the low temperature crystallographic studies of photolytic states were carried out on different Mb mutants and/or at different temperatures (Brunori et al., 2000; Chu et al., 2000; Ostermann et al., 2000). Novel results showed that the photolyzed ligand was migrating away from the distal pocket into the Xe cavities, initially populating the Xe4 cavity on the distal side; however, as temperature was increased from ultra-low to above the solvent glass transition, the photolyzed CO migrated to the other prominent Xe1 cavity on the proximal side. The sophistication has gone so far as to follow the CO dynamics inside and in between cavities by temperature derivative infrared spectroscopy (Lamb et al., 2002; Kriegl et al., 2003).

All these experiments showed that the photodissociated CO momentarily trapped in the matrix was found either on the distal side near the heme iron (*primary docking site*) or in the two Xe binding cavities on the distal and the proximal sides (called *secondary docking sites*) (see Brunori and Gibson, 2001; Schlichting and Chu, 2000 for reviews), substantiating the interpretation of the effect of Xe on geminate rebinding (Brunori et al., 1999; Scott and Gibson, 1997). Although at ultra-low temperatures the motions of the globin are essentially arrested, migration of the photolyzed CO was reported (Brunori et al., 2000; Chu et al., 2000; Ostermann et al., 2000); as the temperature was increased longer range migration was observed, coupled to structural changes and conformational flexibility (see above). These results led to the presumption that also at room temperature a pathway for migration of the ligand inside the protein involves the pre-existing Xe cavities, providing one possible explanation for the complexity of the geminate recombination. However, a direct demonstration of the role of Xe cavities in ligand migration required real time-resolved crystallographic data of high quality.

7. Time-resolved crystallography

The pioneering work of Moffat and collaborators (Srajer et al., 1996) paved the way to room temperature time-resolved crystallography using Laue diffraction. The experimental approach is in principle quite simple. A crystal of MbCO placed in the white X-ray beam is hit with a powerful and very short laser pulse (nanosecond to picosecond) to induce the rupture of the Fe(II)–CO

bond. The photodissociation sets into motion the reorganization of the protein and the migration of the ligand, which either recombines or eventually escapes to the bulk. The structural changes of the protein after photodissociation can be followed by interrogating the crystal with a short pulse of X-rays (approx. 150 ps), as obtained at the ESRF synchrotron source using the single bunch mode. Thus, structural information can in principle be obtained at high resolution by X-ray diffraction of the crystal, at different times after photolysis.

In practice, such type of experiment is extremely demanding in terms of technology, data analysis and quality of the protein crystal. Srajer et al. (1996) in their heroic effort to provide the first time-resolved crystallographic data on photolyzed MbCO, proved that the experiment was feasible. However, it took a great deal of additional work and several important technical improvements (Bourgeois et al., 2000) to achieve the quality of the diffraction data necessary to directly attack the problem of the kinetics of the conformational changes of the globin set into motion by photodissociation.

In the last years, three papers with novel information concerning the structural dynamics of Mb have been published (Bourgeois et al., 2003; Schotte et al., 2003; Srajer et al., 2001). Mbs from two species and different site-directed mutants were employed to obtain high quality data (up to 1.55 Å resolution), extending from picosecond up to millisecond, which allowed a correlation with the ultra-low temperature crystallographic experiments mentioned above. In particular, the results published by Bourgeois et al. (2003) were so good that the relaxation of the globin moiety from nanosecond to millisecond, and the pathway of the ligand migrating inside the protein, could be followed with confidence. Comparison with earlier experiments on ''frozen'' intermediates and with molecular dynamics simulations (now extended to overlap the experimental data) is most revealing.

8. A molecular movie

The paper by Bourgeois et al. (2003) led to novel results because of several reasons. Substantial improvements in synchrotron technology and data analysis were essential. High quality crystals could be obtained with the triple mutant Mb-YQR (see above) because this material proved very sturdy and crystallized very well in the presence of CO to produce the carbon monoxide derivative. Moreover, Mb-YQR is characterized by the absence of significant geminate CO rebinding and by a bimolecular combination rate constant which is 10 times slower than wt Mb (Brunori et al., 1999), thereby extending the time window over which conformational changes can be monitored.

The new time-resolved study shows that, after photolysis, displacement of the heme iron towards the proximal His93(F8), heme tilt, and Tyr29(B10) side chain swinging motion to reach the deoxy conformation are fully developed within 3 ns (see Fig. 3) and decay only at the longest delay times, as CO rebinds. On the same time scale, docking of photodissociated CO in the Xe_4 site, lined (among others) by Tyr29(B10), is observed. On a time scale which is two orders of magnitude slower we observed the globin response ensued by ligand dissociation, similar to the propagation of a ''protein quake'' as defined by Ansari et al. (1985), from the focal point toward the periphery. Thus maximal achievement of the deoxy conformation occurs at approx. 300 ns, showing a twist of the E-helix, to which the motion of Glu64(E7) is largely slaved. A displacement of the CD-turn accompanies the twisting motion of the E-helix, as a result of a mutual steric hindrance between Phe46(CD4) and Asp60(E3). This repulsive interaction may be viewed as the coordinator of the movements of helix E and of the CD-turn. Synchronous with these slower and more extended motions, CO disappears from the Xe4 cavity on the distal side and populates the Xe1 proximal cavity (see below). A *molecular movie* is available as supplementary material online.

The separation in time of motions of different parts of the protein in response to the photolytic event is the first structural interpretation for the stretched time-course of globin relaxation to deoxy Mb, and thus of the complex protein energy landscape (Frauenfelder et al., 2003).

9. The globin relaxation

Time-resolved spectroscopy in solution has provided extremely accurate data to characterize the kinetics of the relaxation to deoxy Mb after photolysis of CO. Many groups have contributed to our present understanding of the dynamics of Mb, using different spectroscopic approaches. Lim et al. (1993) reported that the relaxation to the deoxy Mb optical spectrum after picosecond photodissociation of CO follows a stretched time course; the changes in absorbance at 760 nm (corrected for thermalization) indicated that relaxation initiates at $\sim$10 ps after photolysis and continues up to almost ms. Analysis of the small shift in this absorption (called band III) should be confronted with more recent data (Nienhaus et al., 2002) which suggested that ligand migration is associated to this shift. These results, together with many other experiments, showed that the deoxy Mb relaxation, short of being described by a single exponential, indeed demands a stretched exponential representation, consistent with the hypothesis that the protein relaxes over a very complex time-energy domain.

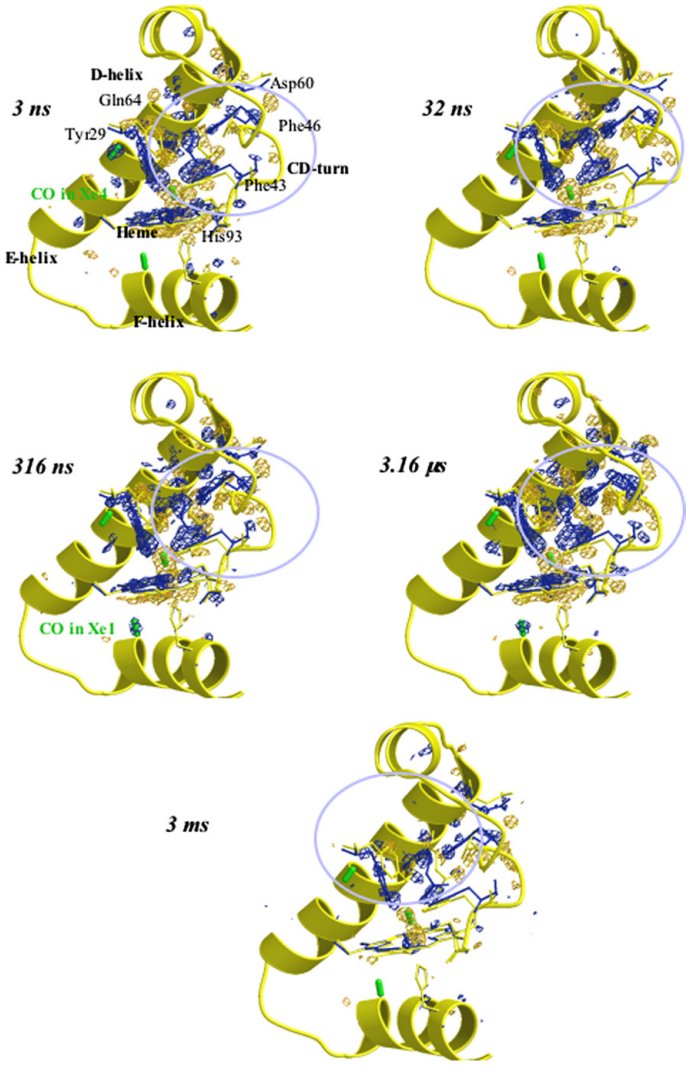

Fig. 3. Time-resolved Laue crystallography: detailed view of the time-dependant electron density differences (yellow: negative; blue: positive, contoured to +/− 3.5 σ) overlaid on the models of YQR-MbCO (yellow) and YQR-$Mb_{316\,ns}$ (blue). In the heme vicinity, the disappearance of bound CO, tilting of the heme, swinging motion of Tyr29(B10) and migration of photolyzed CO to the Xe4 docking site (shown in green) are fully developed by 3 ns. By 316 ns, the photolyzed CO has reached the Xe1 docking site, in concert with the motions of the E-helix, CD-turn, and particularly those of Asp60(E3) and Phe46(CD4) (located inside the blue ellipse). No significant motion is observed around the F-helix.

Extensive geminate recombination studies, carried out with different ligands (CO, O_2, and NO) as a function of temperature and viscosity, provided information on the reactivity of the geminate states, vital to assess the functional significance of dynamics to physiology. The time dependence of ligand rebinding (see Ansari et al., 1994) was assumed to imply a time-dependent inner barrier, imposing a large change in geminate rate constant, which for CO drops by approx. 10^3-fold. It was concluded that time-dependent changes in the structure of deoxy Mb, though small, are associated to large changes in reactivity, and thus are very significant to control the biochemistry of ligand binding (and by inference the efficiency of O_2 delivery).

Spectroscopy, ideal as it is for kinetic analysis, provides no information on the relaxation of the overall globin structure in going from liganded to deoxy Mb. The time-resolved crystallographic data summarized above have shown, for the first time, that the relaxation of the globin after photolysis of MbCO follows an extended time course (Bourgeois et al., 2003), with conformational changes in the immediate vicinity of the heme occurring below nanosecond and those involving secondary structures extending towards millisecond. The parallel work by Schotte et al. (2003) reported that the initial structural changes of His64(E7) seen at 150 ps, have a magnitude greater than expected (Schotte et al.).

Such a correlation between spectroscopy and structure is relevant to assess the role of conformational substates of the protein for function. The extended dynamics of the internal motions seen in Mb-YQR supports the idea that the protein populates different

conformational substates, and that internal motions occur at different rates. The physical basis of this complex time dependence is as yet uncertain, but it may be envisaged that the heterogeneous nature of the protein core is associated to a heterogeneous friction of the protein interior, spreading the relaxation time of the internal conformational changes. Because crystallography yields information on the mean structure of all units in the crystal, it is likely that the delayed structural changes arise from evolution in the relative occupancies of conformational substates (Hagen and Eaton, 1996).

10. Migration of the ligand

Room temperature crystallographic data outlined above are consistent with the results obtained by ultra-low temperature crystallography. In the case of wild type Mb, the photodissociated CO resides initially in the distal pocket (*primary docking site*), and then tends to migrate to the Xe_1 cavity, on the proximal site of the heme; this long trajectory inside the protein takes a few hundred nanoseconds (Srajer et al., 2001). The experiments carried out on two different mutants (Bourgeois et al., 2003; Schotte et al., 2003), both containing an aromatic residue at position B10, are also consistent with cryo-crystallography, indicating that initially CO populates the distal Xe_4 cavity and subsequently migrates to the Xe_1 site, reaching a peak at approx. 0.5 μs (or above) (Fig. 4). Thus, it seems clearly established that the photolyzed ligand (which in the crystal is between 20 and 40% of the total) migrates inside the protein following a pathway that leads from the distal side to the proximal side through a series of connected cavities. The time range of this internal migration coincides with the overall globin relaxation.

The demonstration that the specific and heterogenous internal structure of the protein dictates the pathway for migration and controls the position of the ligand relative to the iron, has some consequences of interest. High quality crystallographic data can now be compared with molecular dynamic simulations that have been extended to overlap in time with the experiments. Bossa et al. (2004) reported an extended simulation (>90 ns) on wild type Mb, which is in agreement with available experimental data, both in terms of pathway of migration of the ligand and structural changes associated to the protein relaxation. A unique value of this simulation is an assessment of the driving force behind the migration of CO along a specific internal pathway; moreover, the simulation shows that the diatomic ligand enhances the probability of opening of the cavity-connecting

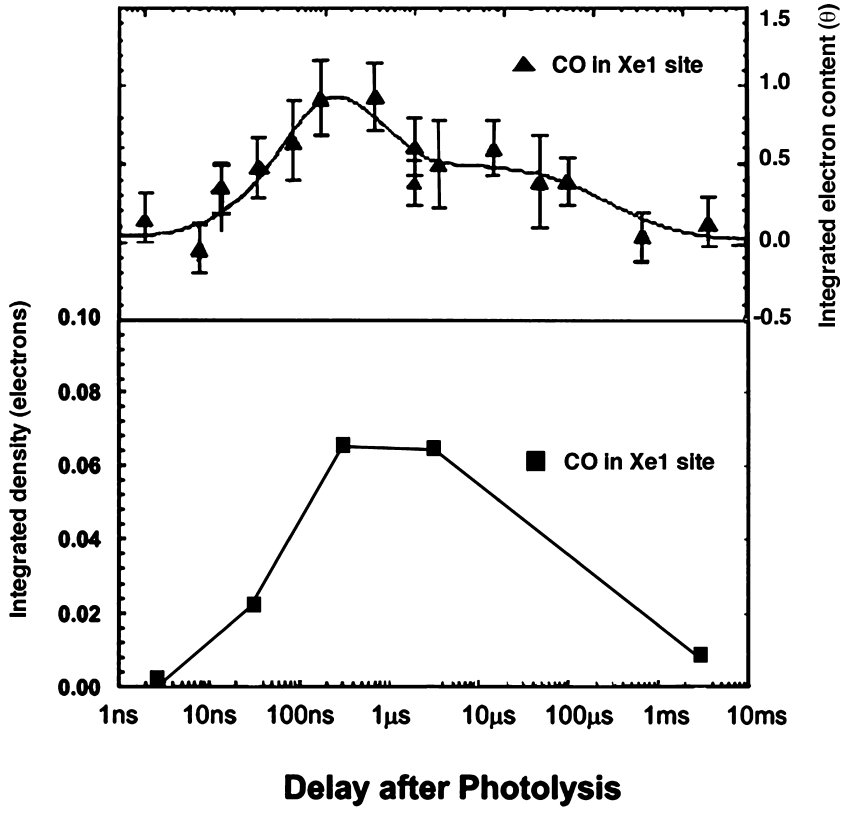

Fig. 4. Time course of CO migration to the Xe1 cavity for wt Mb (data from Srajer et al., 2001 on top) and Mb-YQR (data from Bourgeois et al., 2003 on bottom, modified).

channels. Finally, the agreement provides support to the crystallographic data which are often subject to criticisms because of possible spurious effects due to crystal contacts.

11. Control of reactivity by internal dynamics

A popular mechanism implies that the rates of geminate rebinding from within the protein are correlated with the diffusion of the ligand through the matrix. The existence of temporary *docking sites* for CO seems to agree with a branched model for geminate recombination (Brunori and Gibson, 2001, Scott and Gibson, 1997) and provides a structural interpretation for two related phenomena, i.e., the control of function by the internal structure and the role of the cavities in more complex chemistry "catalysed" by Mb and recently brought to focus.

It has already been pointed out that the extended protein relaxation, encompassing a time range from picosecond to almost millisecond, is associated to a decrease in ligand rebinding; this phenomenon has a direct effect on ligand affinity and thus on biological function. Along the same lines, it was proposed that rapid fluctuations and affinity for O_2 are correlated, the rate of overall ligand dissociation which determines efficiency of delivery, being controlled by three factors, i.e.: the barrier for rupture of the coordination bond between the ligand and the iron; the role of nearby residues in stabilizing the bound ligand (classically the H-bond between His(E7) and the bound O_2, see above); and the temporary sequestration of the ligand in the cavities and restriction of escape into the bulk. Brunori et al. (1999a) presented an hypothesis based on the idea that regulation of function and thus efficiency of O_2 transport may be achieved by controlling the access to channels connecting the different docking sites.

This type of consideration may be extended over-and-above straight O_2 transport, assuming that dynamics may be involved in controlling the reaction of NO with bound O_2 to form met-Mb and nitrite, which in some way promotes Mb to the rank of a "catalyst" (Brunori, 2001). The pathophysiological significance of this new function of Mb, which emphasizes its role as a NO scavenger, has been substantiated by experiments carried out in vivo using Mb knock-out mice (Flögel et al., 2001). Since the reaction of NO with MbO_2 yields nitrate by collision with O_2^- bound to a formally ferric heme iron, it was proposed (Brunori and Gibson, 2001) that temporary sequestration of NO into the cavities may enhance the efficiency of this process by increasing the number of *hits* with the iron bound O_2^- per unit time. This example, subject to experimental verification, is useful to illustrate the idea that a protein may be viewed as a "reactor," with the matrix playing a role in controlling the chemistry and the efficiency of more complex reactions (Frauenfelder et al., 2003).

12. Next steps in structural dynamics

The next challenge in time-resolved crystallography is the investigation of the structural dynamics of tetrameric hemoglobin, especially in the T allosteric state (Monod et al., 1965). This is an extremely difficult experiment, of some significance for two reasons. First of all, it may provide information on the structural relaxation of the globin when the tetramer is in the T quaternary state, which may be of great interest for our understanding of the allosteric behaviour of Hb and thus of cooperativity, given that Mb's reactivity is more similar to R-state Hb (Antonini and Brunori, 1971; Eaton et al., 1999; Hopfield et al., 1971). Second, it may be interesting to test whether internal migration through cavities has a role in supporting the mechanism of transport of NO by Hb. It was initially proposed by Stamler and collaborators (Jia et al., 1996) that this physiologically relevant function of Hb proceeds by reaction of NO with the sulphydryl group of Cys(F9)93 of the β chains, yielding Hb-SNO. This mechanism implies that in T-state Hb, NO should migrate from the distal heme pocket of the β chains to the proximal side to react with Cys(F9)93, and thus it demands that a competent dynamic pathway analogous to that observed for Mb (involving the Xe-binding cavities) is available also in Hb. A test of this novel and physiologically important function of Hb may come from structural dynamics experiments of photolytic intermediates of T-state Hb, similar to those illustrated above for Mb. An initial experiment by cryo-crystallography of R-state Hb has been already published (Adachi et al., 2003), but more challenging and informative will be real time-resolved Laue experiments, now in progress.

Over the last 10 years, a number of new and quite different globins have been identified in bacteria, unicellular eukaryotes, plants, and even mammalian tissues (Bolognesi et al., 1997; Burmester et al., 2000; Hargrove et al., 2000; Wittenberg et al., 2002). The structure of some of these proteins has been elucidated, but understanding their physiological role remains a conundrum. A case in question is neuroglobin (Ngb), a globin expressed in the central nervous system with a peculiar topological distribution (Burmester et al., 2000). This protein was shown to be involved in protecting the brain from hypoxic injuries, and in fact its overexpression is beneficial in the recovery from stroke (Sun et al., 2003). However, the function of Ngb is at present poorly understood, some light being shed by the availability of the structure of met human and murine Ngb (Pesce et al., 2003; Vallone et al., 2004). In fact Ngb is characterized by a "canonical" globin fold, but displays some unusual

structural features such as: (i) hexacoordination of the (ferric and ferrous) heme iron in the unliganded state, the sixth position being occupied by the distal histidine and (ii) access to the active site provided by a huge tunnel which opens at the level of a very mobile EF corner, with accessibility to a large cavity ($\sim$280 Å^3) that embraces the heme distally and proximally. These features configure the presence in the Ngb structure of a large ''reaction chamber'' which can trap ligands in a closed environment, the trigger for opening and closure being coupled to the release and capture of heme ligands. These peculiar structural features point towards some catalytic role of Ngb towards small ligands in the nervous system.

In conclusion, we have discussed the concept that over-and-above their significance in protein stability and flexibility, internal cavities may play a functionally important role in controlling reactivity, thereby conferring a selective advantage to these packing defects. In essence, Evolution seems to have taken advantage of these structural imperfections.

Acknowledgments

Work partially supported by a grant from the Ministero dell'Istruzione, Università e Ricerca of Italy (COFIN 2003 to Maurizio Brunori, ''Dinamica strutturale di metalloproteine'').

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