Kinetic Pathways and Barriers for Ligand Binding to Myoglobin*

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Myoglobin is a small globular heme protein that increases the aerobic capacity of striated vertebrate muscle cells by taking up oxygen from blood during rest and delivering O2 to mitochondria during muscle contraction when blood flow through capillaries is restricted. The ferrous form of myoglobin can also react with CO and NO, which are produced in vivo as second messengers for regulating various physiological functions including blood pressure, platelet aggregation, and neurotransmission. Its tertiary structure consists of eight tightly packed helices, and the resulting “myoglobin fold” is very similar to that found for the α and β subunits of hemoglobin.

Since Gibson’s minireview in 1989, a number of exciting new studies have led to detailed molecular mechanisms of myoglobin function. The rapid progress made in the past 7 years is primarily the result of ultrafast kinetic measurements, mutagenesis experiments, and theoretical molecular dynamics simulations. Significant contributions have been made individually by these approaches, but more progress has occurred when these endeavors have been combined (e.g. Gibson et al., 1992; Braunstein et al., 1993; Schlichting et al., 1994; Teng et al., 1994; Huang and Boxer, 1994; Petrich et al., 1994; Carlson et al., 1994; Quillin et al., 1995). This review focuses on NO, O2, and CO binding to myoglobin mutants under physiological conditions. The objectives are to summarize successes in correlating theoretical, structural, and kinetic results and to identify the major remaining questions in ligand binding dynamics.

Kinetic Schemes and Free Energy Barriers

Springer et al. (1994) have reviewed the stereochemical mechanisms that govern O2 and CO affinity in myoglobin (Mb) and hemoglobin. The kinetics of ligand binding are less well understood and involve multiple steps and stereochemical processes. Oxygen binding to deoxymyoglobin requires: 1) displacement of a non-coordinated, distal pocket water molecule, 2) in-plane movement of the iron atom to form the hexacoordinate complex; and 3) formation of a hydrogen bond between N of His-64(E7) and the second bound O atom.

In laser photolysis experiments, the iron-ligand bond is first disrupted by an intense excitation pulse, producing free ligand inside the distal pocket and pentacoordinate heme with spectral characteristics similar to deoxy-Mb (Fig. 1, upper right). This photodissociation process is seen as a rapid increase in the fraction of deoxy-Mb present during the laser pulse (Fig. 2). The ligand can then either rebind rapidly by an intramolecular process, decreasing the fraction of Mb present, or migrate out into the aqueous phase, leaving a residual fraction of deoxy-Mb. Recombination from solute is a much slower bimolecular process, which is not seen on the picosecond or nanosecond time scales shown in Fig. 2, and its rate is proportional to the total ligand concentration in the solution.

These kinetic phenomena are most often interpreted in terms of the following consecutive reaction scheme.

\[
A \text{ or } MbX = [B_1, \ldots, B_n] = [C_1, \ldots, C_m] = X + Mb \\
\text{ground state} \text{ contact pair} \text{ distal pocket?} \text{ free ligand} \text{ (ps) (ns) (ms)}
\]

**SCHEME 1**

The B intermediates were originally assigned to ligands very close to the iron atom (e.g. contact pairs) and the C states to ligands farther away in the protein matrix. More recent experiments suggest that this distance interpretation is not valid (Carver et al., 1990; Lim et al., 1995b; Carlson et al., 1996). Our current view is that the B states represent a number of ligand positions in the distal pocket with differing rates of approach to the iron atom. In the C or nanosecond state, the ligand is rapidly interconverting between these locations.

Under physiological conditions, intramolecular recombination on nanosecond time scales is usually interpreted in terms of a simple two-step scheme involving either rebinding to form the original liganded complex or ligand escape to the solvent to generate the equilibrium form of Mb (Henry et al., 1983; Carver et al., 1990). These kinetics can be visualized by the free energy diagrams shown in Fig. 3. The barriers and wells were computed from experimentally determined rate constants.

**Differences in Reactivity of Ferrous Ligands**

It has been known for over 75 years that oxygen and nitric oxide react much more rapidly with myoglobin and hemoglobin than CO. The overall association rate constants for CO, O2, and NO binding to native sperm whale myoglobin are 0.5, 17, and 22 μM⁻¹ s⁻¹, respectively. The gaseous recombination time courses in Fig. 2 and the barrier diagram in Fig. 3 provide quantitative interpretations of the differences in kinetic behavior of the diatomic gases.

NO is extremely reactive with ferrous iron, shows little or no inner quantum mechanical barrier to bond formation, and rebinds to native myoglobin almost completely on picosecond time scales. There is no discrete nanosecond intermediate for MbNO, and a continuum of rebinding phases is observed, extending from picoseconds to nanoseconds (Fig. 2A) on gward et al., 1988; Petrich et al., 1991; Gibson et al., 1992). Biomedical NO binding is governed exclusively by the outer kinetic barrier for ligand movement into the distal pocket. Once inside the protein, NO reacts so quickly with the iron atom that escape back into the solvent almost never occurs (Fig. 3).

Quantum mechanical barriers inhibit geminate rebinding of O2 and CO to myoglobin. O2 is moderately reactive on nanosecond time scales. Photolysis of native MbO2 shows ~50% geminate recombination (Fig. 2B), implying that the inner kinetic barrier is roughly equal to that for ligand escape (Fig. 3). CO is the least reactive diatomic gas, has an inner kinetic barrier to bond formation that is almost twice the height of that for ligand escape (Fig. 3), and, as a result, shows little geminate recombination at room temperature (Fig. 2B).

**Ultrafast Spectral Intermediates**

Hochstrasser, Martin, Magde, and co-workers have shown that short-lived spectral intermediates are generated immediately after photolysis of O2 and NO derivatives of both myoglobin and hemoglobin (Cornelius et al., 1981; J'gward et al., 1988; Petrich et al., 1988; Walda et al., 1994). In contrast, no intermediates are observed when either HbCO or MbCO is photolyzed with ultrashort excitation pulses. For NO and O2 complexes, a species designated Mb²⁺ or Hb²⁺ is formed in femtoseconds and has an unusual absorption spectrum. Most of this intermediate decays back to the original liganded ground state within ~2 ps after photolysis, accounting for...
an ~80% reduction in the quantum yield of photodissociated states.

The structural origin of the room temperature Mb* species and its relevance to thermal dissociation and association processes remain unclear. Petrich et al. (1991) examined changes in heme coordination geometry using molecular dynamics simulations. They reported that, within 1–2 ps after photolysis, the iron atom moves out of the plane of the pyrrole nitrogen atoms and takes a position very similar to that found at equilibrium. Additional fluctuations appear to occur in the next 10–1000 ps, but these are minor. Similar results were obtained in molecular dynamics simulations carried out by Gibson et al. (1992). These analyses suggest that the ultrafast (100 fs to 2 ps) phases may represent O2 and NO rebinding to thermally excited, in-plane, five-coordinate heme. CO is too inert to recombine before the iron atom relaxes to its less reactive, out-of-plane position. However, this interpretation is tentative.

**Initial Movement of Dissociated Ligands (10–500 ps)**

Three lines of indirect evidence have suggested that dissociated ligands initially move toward the interior of the protein molecule (Fig. 1). First, Sassaroli and Rousseau (1986) used molecular dynamics simulations to show that there is an energy minimum when dissociated CO moves into the cavity circumscribed by Leu-29(B10), Leu-32(B13), Phe-43(CD1), Val-68(E11), and Ile-107(G8) (Fig. 1). Upper left panel). Sophisticated simulations of NO rebinding to wild-type and mutant myoglobins have come to a similar conclusion (Gibson et al., 1992). Second, the side chain carbon atoms of covalently bound ethyl isocyanide are located in this pocket, which is presumably the sterically most accessible space (Fig. 1, lower left panel). Carver et al. (1990) argued that the ethyl isocyanide complex of myoglobin serves as a model for the initial trajectory of the diatomic gases with the C–N atoms mimicking the position of bound O2, NO, and CO and the CH3CH2– group representing the major position of diatomic ligands in picosecond and nanosecond intermediates. Third, these interpretations are supported by the effects of distal pocket substitutions on picosecond time courses for NO rebinding (e.g. Carver et al., 1990; Gibson et al., 1992; Petrich et al., 1994; Quillin et al., 1995).

These proposals have been verified by the structure of the initial photoproduct for MbCO, which was determined by x-ray crystallography at 20 K (Schlichting et al., 1994; Teng et al., 1994) (Fig. 5, middle). There appears to be a recoil of the ligand after photolysis causing the CO molecule to move ~2 Å away from the iron atom toward the protein interior. It lies parallel to the heme plane in a position very similar to that of the alkyl side chain in ethyl isocyano-myoglobin and that of the non-covalently bound water molecule in the distal pocket of deoxymyoglobin (Fig. 4, bottom and Fig. 5, right). In room temperature experiments using polarized excitation and IR detection, Lim et al. (1995b) have also shown that CO is rotated ~90° in the initial photoproduct relative to its position in the equilibrium bound state.

**The Innermost Kinetic Barriers (10–500 ps)**

The effects of mutagenesis, the structure of the MbCO photoproduc, and molecular dynamics simulations have suggested that picosecond recombination is governed primarily by quantum mechanical restrictions that determine ligand reactivity, secondarily by distal pocket features that enhance or restrict ligand movement, and thirdly by proximal effects that govern the reactivity and/or accessibility of the iron atom. Rapid NO recombination can be achieved in three ways: 1) increasing the reactivity of the metal atom by replacing iron with cobalt (Ikeda-Saito et al., 1993); 2) inhibiting ligand movement away from the iron atom by placing phenylalanine or tryptophan diffusional barriers at positions 29 and 68; and 3) removing steric restrictions directly adjacent to the iron atom (Carlson et al., 1994). The latter experiments suggest that His-64(E7) and perhaps Val-68(E11) push the ligand toward the back of the distal pocket immediately after photolysis. This steric hindrance effect is enhanced markedly when Val-68(E11) is replaced with Ile. (Fig. 2A, V68I trace, and Fig. 3) (Quillin et al., 1995).
Amino acids at the solvent interface are shown in sperm whale myoglobin. The view is looking down onto the heme from the N helix (see Fig. 1). The heme group is shown in dark purple underneath the protein residues. Amino acids at the solvent interface are shown in light blue and those in the protein interior are shown in gray. Bound CO and ethyl isocyanide are shown in red. Upper left, wild-type MbCO (Quillin et al., 1993); upper right, V68F MbCO (Quillin et al., 1995); lower left, wild-type Mb-ethyl isocyanide (Eich et al., 1996); and lower right, V68F Mb-ethyl isocyanide (R. D. Smith, unpublished data).

The photodissociated ligand is forced to take a position parallel to the heme plane by the C-atom, and NO re-binding is extremely rapid (Fig. 2 with Phe). Most of the ligands reflect back immediately to the iron atom. However, once the ligand moves past this residue to the back of the pocket, geminate recombination will affect the rate of non-covalent ligand binding regardless of the exact pathway for movement into the protein. The three-dimensional structure of myoglobin reveals no pathways or channels for O2 entry and exit. Transient motions of the protein are required to

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**Fig. 4. Distal pocket structures of wild-type and V68F mutants of sperm whale myoglobin.** The view is looking down onto the heme from the N helix (see Fig. 1). The heme group is shown in dark purple underneath the protein residues. Amino acids at the solvent interface are shown in light blue and those in the protein interior are shown in gray. Bound CO and ethyl isocyanide are shown in red. Upper left, wild-type MbCO (Quillin et al., 1993); upper right, V68F MbCO (Quillin et al., 1995); lower left, wild-type Mb-ethyl isocyanide (Eich et al., 1996); and lower right, V68F Mb-ethyl isocyanide (R. D. Smith, unpublished data).

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**Fig. 5. Crystal structures of wild-type MbCO (left), photodissociated MbCO (middle), and deoxy-Mb (right) at 20 K in the P6 form.** The electron density maps (Fo – Fc) were taken from Schlichting et al. (1994). The iron electron density is shown in red. The red square encloses, from left to right, bound CO, photodissociated CO, and non-coordinated distal pocket water. The iron atom is, from left to right, in the plane of the pyrrole nitrogens, partially out-of-plane, and completely out-of-plane, respectively (see Fig. 1).
allow access to the iron and net dissociation to the surroundings. Two distinct mechanisms have been proposed for ligand movement into and out of myoglobin. The first involves rotation of the side chain of His(E7) to create a direct channel from the solvent to the iron atom (see Perutz, 1989) (Fig. 1). The evidence in favor of this hypothesis includes: (a) outward displacement of His-64 in the crystal structures of the imidazole, phenylhydrazine, and ethyl isocyanide complexes of native sperm whale myoglobin (Bolognesi et al., 1982; Ringe et al., 1984; J. Dhonson et al., 1989); (b) increases in rates of ligand binding following protonation and outward movement of His-64 at low pH (Tian et al., 1993; Yang and Phillips, 1996); and (c) increases in the rate constants for ligand entry when Phe-46 is replaced with Val or Ala causing disorder in the position of His-64 (Lai et al., 1995).

The second hypothesis is that ligands enter and leave the distal pocket through several longer hydrophobic pathways between the B, G, and H helices. First proposed by Elber and Karplus (1990) on the basis of molecular dynamics simulations, this interpretation has been adopted by Huang and Boxer (1994) to explain their random mutagenesis data. In the latter experiments, point mutations at positions far removed from the distal pocket exert large effects on the rates for CO and O2 binding. This idea is also consistent with the rate of ligand escape being roughly the same, \( \sim 10^5 \text{ s}^{-1} \), for almost all of the point mutations examined so far and with the presence of multiple xenon binding sites in the interior of myoglobin (Tilton et al., 1984, 1988).

**Future Prospects**

There is a clear need for further characterization of the ultrafast spectral intermediates seen after femtosecond photolysis of MbO2 and MbN0. Identification of the chemical nature of these species will require the use of mutants, model compounds, time-resolved vibrational spectroscopy as well as conventional kinetic measurements, and quantum mechanical descriptions of photoexcitation and bond formation. A description of ligand movement into and out of myoglobin will require simulations on nanosecond time scales, time-resolved IR analyses of photodissociated CO in a wide variety of mutant myoglobins, and x-ray crystallographic structure determinations of nanosecond intermediates.

Perhaps the most exciting prospect is the application of these biophysical studies to the rational design of more stable and efficient O2 delivery pharmaceuticals. Recently, Eich et al. (1996) have used Leu-B10 \( \rightarrow \) Phe and Val-E11 \( \rightarrow \) Phe mutations to limit NO-induced oxidation of bound O2 in both myoglobin and hemoglobin. These substitutions were chosen because they had previously been shown to inhibit ligand entry into the distal pocket. Similar strategies are being applied to inhibit autooxidation and to enhance O2 transport and discrimination against CO binding.

Thus, the study of myoglobin function remains an active and important research field. In addition to providing greater understanding of the physiology of O2 delivery, this work provides a direct connection between molecular dynamics simulations and ultrafast kinetic and structural measurements. This linkage between theory and experiment can now be used interactively to develop new theoretical approaches and strategies for protein engineering.

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**REFERENCES**

Ansari, A., Jones, C. M., Henry, E. R., Hofrichter, J., and Eaton, W. A. (1994) Biochemistry 33, 5128–5145

Bolognesi, M., Cannillo, E., Ascenzi, P., Giaconetti, G. M., Merli, A., and Brunori, M. (1982) J. Mol. Biol. 158, 305–315

Braunstein, D. P., Chu, K., Egeberg, K. D., Frauenfelder, H., Mourot, J. R., Nienhaus, G. U., Ommor, P., Sligar, S. G., Springer, B. A., and Young, R. D. (1982) J. Mol. Biol. 158, 305–315

Carlson, M. L., Regan, R. L., Li, H., Phillips, G. N., Jr., Olson, J. S., and Gibson, Q. H. (1994) Biochemistry 33, 10597–10606

Cornelius, P. A., Regan, R. M., and Gibson, Q. H. (1989) J. Chem. Phys. 91, 2765–2775

Elber, R., Li, H., Regan, R., and Gibson, Q. H. (1995) J. Mol. Biol. 250, 2665–2677

Elber, R., and Karplus, M. (1990) J. Am. Chem. Soc. 112, 9161–9175

Fourenfelder, H., Park, F., and Young, R. D. (1988) Annu. Rev. Biophys. Biophys. Chem. 17, 451–479

Gibson, Q. H. (1989) J. Biol. Chem. 264, 20155–20158

Gibson, Q. H., Regan, R. L., Elber, R., Olson, J. S., and Carver, T. E. (1992) J. Biol. Chem. 267, 22022–22034

Henry, E. R., Sommer, J., Hofrichter, J., and Eaton, W. A. (1983) J. Mol. Biol. 166, 443–451

Huang, X., and Boxer, S. G. (1994) Nat. Struct. Biol. 1, 226–229

Ikeda-Saito, M., Dou, Y., Yonetani, T., Olson, J. S., Li, T., Regan, R., and Gibson, Q. H. (1993) J. Biol. Chem. 268, 6855–6857

Johnson, K. A., Olson, J. S., and Phillips, G. N., Jr. (1989) J. Mol. Biol. 207, 1125–1136

Jongewaard, K. A., Magde, D., Tauke, D. J., Marsters, J. C., Traynor, T. G., and Sharma, V. S. (1988) J. Am. Chem. Soc. 110, 380–387

Lai, H. H., Li, T., Lyons, D. S., Phillips, G. N., Jr., and Olson, J. S. (1995) Proteins Struct. Funct. Genet. 22, 327–339

Lim, M., Jackson, T. A., and Anfinrud, P. A. (1995a) J. Chem. Phys. 107, 4355–4366

Lim, M., Jackson, T. A., and Anfinrud, P. A. (1995b) Science 269, 962–966

Ormos, P., Braunstein, D., Frauenfelder, H., Hong, M. K., Lii, S.-L., Sauke, T. B., and Young, R. D. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 8492–8496

Perutz, M. F. (1989) Trends Biochem. Sci. 14, 42–44

Petrich, J. W., Poyart, C., and Martin, J. L. (1988) Biochemistry 27, 4049–4060

Petrich, J. W., Lambry, J.-C., Kuczerka, K., Karplus, M., Poyart, C., and Martin, J.-L. (1991) Biochemistry 30, 3975–3987

Quillin, M. L., Lambry, J.-C., Brenman, J. W., Chernoff, D. A., and Martin, J. L. (1994) J. Mol. Biol. 238, 437–444

Quillin, M. L., Arduini, R. M., Olson, J. S., and Phillips, G. N., Jr. (1993) J. Mol. Biol. 238, 437–444

Quillin, M. L., Li, T., Olson, J. S., Phillips, G. N., Jr., Dou, Y., Ikeda-Saito, M., Elber, R., Li, H., Regan, R., and Gibson, Q. H. (1995) J. Biol. Chem. 270, 416–436

Ringe, D., Petsko, G. A., Kerr, D., and Ortiz de Montellano, P. R. (1984) Biochemistry 23, 2–4

Sassaroli, M., and Rousseau, D. L. (1986) J. Biol. Chem. 261, 16292–16294

Schlichting, I., Berendzen, J., Phillips, G. N., Jr., and Sweet, R. M. (1994) Nature 370, 808–812

Smerdon, S. J., Dodson, G. G., Wilkinson, A. J., Gibson, Q. H., Blackmore, R. S., Carver, T. E., and Olson, J. S. (1991) Biochemistry 30, 6522–6526

Springer, B. A., Sligar, S. G., Olson, J. S., and Phillips, G. N., Jr. (1994) Chem. Rev. 94, 699–714

Teng, T.-Y., Srajer, V., and Moffat, K. F. (1994) Nat. Struct. Biol. 1, 701–705

Tian, W. D., Sage, J. T., and Champion, P. M. (1993) J. Mol. Biol. 233, 155–166

Tilton, R. F., Jr., Kuntz, I. D., Jr., and Petsko, G. A. (1984) Biochemistry 23, 2849–2857

Tilton, R. F., Jr., Singh, U. C., Kuntz, I. D., Jr., and Kolman, P. A. (1988) J. Mol. Biol. 199, 195–211

Walda, K. N., Liu, X. Y., Sharma, V. S., and Magde, D. (1994) Biochemistry 33, 2198–2209

Yang, F., and Phillips, G. N., Jr. (1996) J. Mol. Biol. 256, 762–774