Accelerated Publications

Reaction of Myoglobin with Phenylhydrazine: A Molecular Doorstop†

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ABSTRACT: X-ray crystallographic studies of myoglobin do not show an entrance or exit path for potential ligands from the surface to the heme cavity. Efforts to locate such a path have so far centered around dynamic calculations. A structure has now been determined that has a clear opening. Phenylhydrazine reacts with myoglobin in such a way that a phenyl group remains bound to the iron atom. The structure of this complex shows that the side chains of His-64(E7), Arg-45(CD3), and Val-68(E11) have been forced aside to form an open channel to the surface. Although this may not be the only channel to the iron atom, it seems likely that it is an important one.

According to the three-dimensional structures of myoglobin determined by X-ray diffraction (Kendrew et al., 1960; Perutz & Matthews, 1966; Nobbs, 1966; Takano, 1977a,b), there is no obvious entrance or exit path for potential ligands from the outside of the protein into the distal heme "pocket". In fact, examination of the structures of deoxymyoglobin and liganded myoglobin has indicated that accessibility of the heme pocket to ligands is blocked by the side chains of several amino acids. Numerous suggestions have been made for structural changes that would form a path to the ligand binding site, many involving fluctuations of parts of the E-helix. For instance, rotation of His-64(E7), followed by movement of the Arg-45(CD3) side chain, would allow entry of a ligand (Nobbs, 1966). However, such a movement has as yet not been observed. Binding of imidazole to metmyoglobin causes extension of the b-axial length in the crystal (Nobbs, 1966). This has been interpreted to indicate that His-64(E7) and Arg-45(CD3) have been forced away from their normal positions to accommodate the ligand. Trajectory and energy minimization calculations have indicated that His-64(E7) and Val-68(E11) lie in broad potential wells (Case & Karplus, 1979). In addition, involvement of Val-68(E11) is implicated by dependence of the methyl NMR on pH, the binding of anions, and the nature of the ligand bound to the iron (Lindstrom & Ho, 1973).

Consequently, two major paths have been postulated, to which the following amino acid residues make the dominant contributions to the energy barriers. For the classical path, His-64(E7), Thr-67(E10), and Val-68(E11) are the important residues. For the secondary path, Leu-61(E4) and Phe-34(B14) are the important residues.

A study of the dynamics of the myoglobin structure by X-ray diffraction (Frauenfelder et al., 1979) shows that none of these residues is particularly mobile but that there is a region of amino acids with relatively large mean-square displacements in the vicinity of and including Arg-45(CD3).

A crystallographic study of methemoglobin with imidazole bound at the iron atom indicates that binding of this ligand requires motion of His-64(E7), Val-68(E11), and Phe-43(CD1) and -46(CD4), as well as movement of the heme toward the proximal side (Bell et al., 1981).

If, as these postulates indicate, various parts of myoglobin fluctuate in order to open a "door" into the interior of the molecule, specifically into the distal heme pocket, then it should be possible to find a ligand that is small enough to fit into the channel and pocket but large enough to hold the channel open like a molecular "doorstop". A structure of myoglobin has now been determined in which such a ligand is bound to the iron, while at the same time forcing the molecule to maintain an open channel to the surface.

Experimental Procedures

Materials. Metmyoglobin crystals were grown from 6% protein solutions in 40% saturated ammonium sulfate, pH 6.4. Phenylhydrazine (2.5 mg, 0.017 mol) was added to 1 mL of 50% saturated ammonium sulfate at pH 6.6. The crystals were washed, transferred to the phenylhydrazine solution, and allowed to soak for 1 week at room temperature. One of the treated crystals was mounted in a quartz capillary for X-ray data collection. A second crystal was washed and then dissolved in water. The ultraviolet absorption spectrum of the treated myoglobin agreed with that reported for the reaction of myoglobin with phenylhydrazine in solution (Kunze & Ortiz de Montellano, 1983).

Methods. Data to 1.5-Å resolution were collected on a Nicolet P3 diffractometer equipped with a modified LT 1 low-temperature device. Measurements were made at 263 K by fully integrating over the entire ω profile of each reflection. Reaction of metmyoglobin with phenylhydrazine caused cracking of the crystals, so a rather wide scan (2.2°) was used. No significant radiation damage was observed at the low temperature. Data were reduced in the usual way (Frauenfelder et al., 1979). A total of 11 531 observed reflections were

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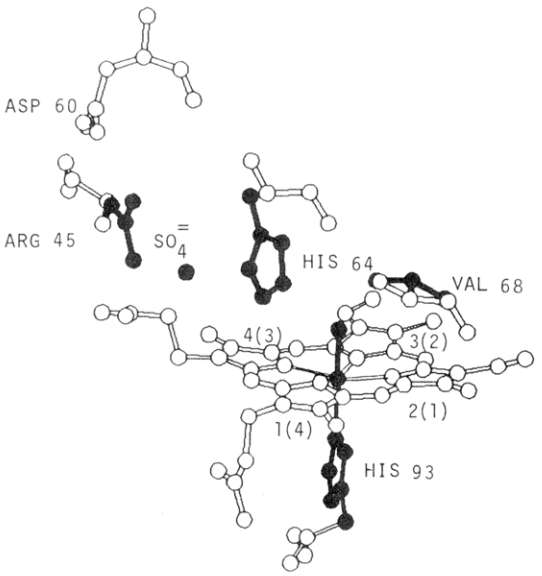

FIGURE 1: Drawing of the residues around the heme in metmyoglobin that are involved in the formation of a channel when phenylhydrazine is added. The iron atom, bound water molecule, sulfate ion, and amino acid side chains undergoing changes are indicated in black. The pyrrole numbering is given according to the two conventions: Protein Data Bank (metalloprophyrin).

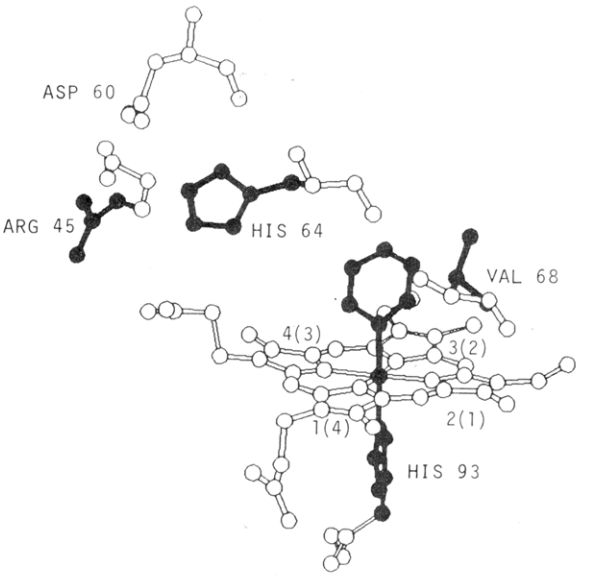

FIGURE 2: Drawing of the residues around the heme in myoglobin with phenyl bound to the iron atom. Asp-60(E3) and Arg-45(CD3) are close enough to each other to form an ion pair. The iron atom, phenyl group, and amino acid side chains, which have moved as a consequence of the phenyl group, are indicated in black.

used to calculate a difference Fourier map using phases and native amplitudes from a refined metmyoglobin structure at 250 K (R = 21%; D. Ringe, unpublished results). The bound phenyl group was apparent, as were a number of changes in the positions of residues near the heme. A $2F_{\text{phenyl}} - F_{\text{native}}$ Fourier map displayed on a Vector General computer graphics system was used to build in the phenyl group and reposition the necessary residues. Finally, the structure of the complex was refined by a restrained least-squares method (Hendrickson & Konnert, 1979) to give a final R factor of 22% with 0.02-Å rms deviation from ideal bond lengths.

Results and Discussion

Formation of an Aryl Heme. In native metmyoglobin, the water molecule at the heme iron is in close van der Waals contact with the methyl groups of Val-68(E11) and is hydrogen bonded to the N^{ϵ} of His-64(E7) (Figure 1). The N^{δ} of His-64(E7) is hydrogen bonded to a sulfate ion, which forms an ion pair with the side chain of Arg-45(CD3). This arginine is also hydrogen bonded to the propionic acid side chain of pyrrole 4(3) (4 refers to the Protein Data Bank convention, and 3 refers to the metalloporphyrin convention for numbering the pyrrole rings) and the carboxylate of Asp-60(E3).

The reaction between metmyoglobin and phenylhydrazine produces a complex in which the phenyl group is coordinated to the iron atom (Figure 2). The reaction in solution causes a spectral shift indicative of this binding (Kunze & Ortiz de Montellano, 1983).

The reaction in the crystal results in a protein sample exhibiting the same shift. The difference Fourier of a crystal treated with phenylhydrazine shows six positive peaks and five negative peaks. One of the positive peaks occurs at the position normally occupied by heme-bound water and represents the phenyl ring. The other five peaks are each associated with a negative peak, indicative of a rearrangement of part of the protein structure.

The phenyl ring is coordinated to the iron atom through one of the ring carbon atoms. The iron–carbon distance is 1.9 Å, which is in agreement with bond distances observed for iron(III)–carbon coordination (Pauling, 1960). The plane of the phenyl ring is perpendicular to the plane of the heme, passing through the iron atom and across the methine carbon between pyrroles 2(1) and 3(2). The orientation of the phenyl group relative to the proximal histidine is such that the two planes form an angle of 50°.

A number of predictable changes have occurred in the structure of the protein. The iron atom has moved 0.3 Å, so that it now lies in the plane of the heme. In so doing, the proximal histidine [His 93(F8)] has moved approximately 0.5 Å closer to the heme in order to maintain a bonding distance between the iron atom and the histidine N^{ϵ}. The F-helix has shifted by 0.15 Å toward the FG corner in the same way as occurs in the transition from deoxymyoglobin to metmyoglobin (Takano, 1977a,b; Dickerson & Geis, 1983). The imidazole ring has turned by 9° away from the $N_{1(4)}$–$N_{3(2)}$ direction.

The Doorstop. A number of unpredictable changes have occurred in the structure of the protein, which produce a structure with a stabilized access channel from the surface to the iron atom. In order to accommodate the phenyl ring in the distal heme pocket, two groups have rotated out of the way. The first of these is the isopropyl group of Val-68(E11). By a rotation of 100° about the C^{α}–C^{β} bond, the methyl groups have made room for the new ligand. The second movement is of the imidazole group of His-64(E7). By a rotation of 100° about the C^{α}–C^{β} bond, this ring system has swung out of the way of the ligand. In so doing, the N^{ϵ} of His-64(E7) has moved 5.4 Å. In both cases, the new position of the group is accommodated by an existing cavity in the structure. The sulfate ion is displaced by the imidazole of His-64(E7) and is not observed in the phenylmyoglobin structure.

Finally, the rotation of the imidazole group of His-64(E7) has forced the side chain of Arg-45(CD3) to rotate outward around the C^{γ}–C^{δ} bond (45°) and the C^{δ}–C^{ϵ} bond (70°), forming a channel from the surface to the interior of the heme pocket. The new arrangement is stabilized by hydrogen bonding between His-64(E7) and Arg-45(CD3) and an ionic interaction between Arg-45(CD3) and Asp-60(E3). The channel thus formed is almost parallel to the heme and follows

a direction from the iron atom, across pyrrole 4(3) of the heme, and out through the space provided between Arg-45(CD3) and the propionyl group of pyrrole 4(3). The position of the propionyl group of the heme [at ring 4(3)] has not changed. However, an increase in the mobility of the carboxyl group in the phenyl-liganded structure, as measured by isotropic temperature factors, is observed, presumably because the carboxyl is no longer stabilized by hydrogen bonding.

The accessible surface areas (Lee & Richards, 1971) of native myoglobin and myoglobin with the channel held open have been determined in order to ascertain the approximate size of the channel. A probe of 1.8-Å radius was used because it represents the approximate size of an oxygen molecule. The calculation showed that the position of coordination to the iron is not accessible in the native structure. Moreover, the "door" to the distal pocket, His-64(E7), is largely inaccessible due to its "closed" position and shielding by the side chain of Arg-45(CD3). For the liganded structure, the calculation was done with the residues in their new conformations. The presence of the phenyl ring would block the probe from making this measurement. Therefore, only the single carbon atom liganded to the iron was retained to serve as a marker for the actual binding site. In this "open" structure, the channel residues, including the complete side chain of His-64(E7) and the ligand position, are available to the probe, indicating that the channel is large enough to allow the passage of an oxygen molecule from the surface to the heme pocket and consequently the iron atom.

This structure of myoglobin, with a channel held open by a molecular doorstop, the phenyl group, may represent the end product of a triggered conformational change caused by the binding of a ligand to the surface of the protein at or near the Arg-45(CD3)–propionyl salt bridge. It indicates one pathway that oxygen or carbon dioxide can take in getting from the surface to the iron atom.

Acknowledgments

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Registry No. Phenylhydrazine, 100-63-0; histidine, 71-00-1; arginine, 74-79-3; valine, 72-18-4.

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