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Short Communication

Organometallic myoglobins: Formation of Fe–carbon bonds and distal pocket effects on aryl ligand conformations

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ABSTRACT

Bioorganometallic Fe–C bonds are biologically relevant species that may result from the metabolism of natural or synthetic hydrazines. The molecular structures of four new sperm whale mutant myoglobin derivatives with Fe–aryl moieties, namely H64A–tolyl-*m*, H64A–chlorophenyl-*p*, H64Q–tolyl-*m*, and H64Q–chlorophenyl-*p*, have been determined at 1.7–1.9 Å resolution. The structures reveal conformational preferences for the substituted aryls resulting from attachment of the aryl ligands to Fe at the site of net $-NHNH_2$ release from the precursor hydrazines, and show distal pocket changes that readily accommodate these bulky ligands.

Arylhydrazines ($ArNHNH_2$; Ar = aryl) occur in nature and are important chemical species in both the agrochemical and pharmaceutical industry [1]. Synthetic hydrazines are also prevalent in the general environment [2]. In some cases, hydrazines are beneficial components of pharmaceutical drugs such as the antidepressant drugs isoniazid and phenelzine, but are also toxic and can act as cancer causative agents [1,2]. For example, the newly FDA-approved drug DUOPA® for Parkinson's disease contains the substituted hydrazine carbidopa with a known hemolytic anemia side effect. The parent hydrazine (NH_2NH_2) is found in tobacco and tobacco smoke [3]. Hydrazines can also be present in certain foods. For example, 4-hydroxymethylphenylhydrazine is generated as a metabolite from agaritine, a food component from the edible commercial mushroom *Agaris bisporus* [4].

Phenylhydrazine is toxic to red blood cells [5], and the hemoglobin-phenylhydrazine reaction has been used to model hemolytic anemia. Interactions of substituted hydrazines with human hemoglobin (Hb) may also result in hemolysis, Heinz-body formation, and degradation of the protein [6]. Related interactions of substituted hydrazines and diazenes (RN=NH) with other heme proteins such as myoglobin (Mb) [7–9], cytochrome P450 [10–12], and catalase [13] have been reported [14]. The generated products often contain heme σ-aryl bonds with direct Fe–C(aryl) linkages (Scheme 1) as determined by UV–vis and NMR spectroscopy. In some cases, an Fe–to–N(porphyrin) migration of the aryl ligand (Scheme 1; right) occurs upon extraction of the heme from the protein, or occurs within the heme protein active site (for cytochrome P450) [14].

Only two X-ray crystal structures have been reported to date for such heme protein σ-aryl species. The preparation and crystal structure of the prototypical wild-type (wt) sperm whale (sw) Mb–phenyl complex, obtained from the reaction of ferric Mb (metMb) with phenylhydrazine, was published by Ringe and Ortiz de Montellano and coworkers in 1984 [7]. The related preparation and crystal structure of the cytochrome $P450_{cam}$–phenyl complex, obtained from the reaction with phenyldiazene, was reported by Poulos and Ortiz de Montellano and coworkers in 1990 [12].

The potential of arylhydrazines to serve as steric probes for heme protein active sites has been evaluated [14]. It is well established that the arylhydrazine reactions with heme proteins proceed via carbon-based radicals [15,16] formed during aerobic reaction with the protein en route to Fe–C bond formation [17]. New X-ray structural data that clarify which aryl C atoms bind to the Fe centers, and information regarding preferred aryl ligand orientations from reactions involving substituted arylhydrazines as a function of active site structure are thus desirable.

We have prepared a representative set of derivatives from the reactions of arylhydrazines with wt and mutant Mbs (Chart 1) to determine the mode of attachment of the substituted aryl groups to the iron centers [18], and the effect of mutating the distal pocket H64 residue on the orientation of the aryl ligands. We chose the H64A mutant to remove distal sidechain H-bonding, and the H64Q mutant to provide an alternate H-bonding capacity in the heme pocket.

The aerobic reaction of sw metMb with phenylhydrazine results in spectral changes in the UV–vis spectrum identical to those reported previously (Supporting Information Fig. S1) [8,9]. The related reaction of the metMb H64A mutant (λ_{max} 408 nm) [19] with *m*-tolylhydrazine

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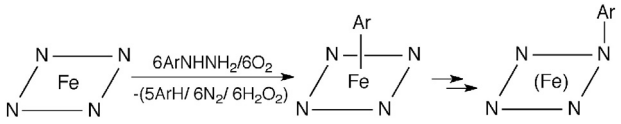

Scheme 1. Formation of σ-aryl (middle) and *N*-aryl (right) products from the reaction of arylhydrazines with heme proteins. The heme is designated by the N_4Fe plane.

in phosphate buffer at pH 7.4 reveal similar spectral features to generate a product (λ_{max} 436 nm), shown in Fig. 1A. When *p*-chlorophenylhydrazine is used for the reaction, similar spectral shifts are observed (Fig. 1B). Both reactions are essentially complete after ~1.5 min (Fig. S2 in the SI). Reactions of the metMb H64Q mutant [19] with *m*-tolylhydrazine and *p*-chlorophenylhydrazine proceed similarly, with the reactions being complete after ~6 min and ~3 min, respectively (Fig. S3 in the SI).

We have successfully obtained the X-ray crystal structures of all four myoglobin H64A–tolyl-*m* (1.77 Å resoln), H64A–chlorophenyl-*p* (1.70 Å resoln), H64Q–tolyl-*m* (1.88 Å resoln), and H64Q–chlorophenyl-*p* (1.87 Å resoln) derivatives. We also reproduced the crystal structure of the known wt sw Mb–phenyl complex [7] at 1.87 Å resolution (Fig. S4 in the SI). Careful exposure of the respective pre-formed metMb crystals to solutions of the arylhydrazines in air resulted in the formation of products that retained their crystallinity.[1] Numerous trials (>40 trials for each derivative) were needed to obtain suitable product crystals with good diffraction, as this reaction generally resulted in disruption of crystallinity and crystal cracking. Indeed, we have not yet obtained suitable crystals for the wt Mb derivatives with the tolyl and chlorophenyl ligands.

The heme sites of the Mb H64A and H64Q products are shown in Fig. 2, and demonstrate direct Fe–C bond formation to generate the product complexes. The Fe–C bond lengths of 1.9–2.0 Å are similar to those obtained in heme model Fe–aryl compounds (~1.96 Å) [20].

The tolyl ligand in the H64A–tolyl-*m* derivative (Fig. 2A) is bound to Fe via the carbon atom bearing the initial hydrazine functional group (i.e., in the *meta* position). The aryl methyl substituent points to the hydrophobic interior of the protein, with the closest contact of 3.4 Å between the ligand methyl C-atom and the δ_2 C-atom of Leu29. The aryl plane essentially eclipses diagonal porphyrin N atoms, and is perpendicular to the heme N_4-plane (with a small ~5° tilt to the heme normal).

In the H64A–chlorophenyl-*p* structure (Fig. 2B), the aryl ligand binds via the *para*-C atom which held the hydrazine moiety. The ligand plane also eclipses diagonal porphyrin N-atoms, but has a larger (~18°) tilt from the normal to the heme plane in the direction of Phe43 and Phe46, with the closest calculated distance being between the centroid of the aryl ring and $C\gamma_2$ of Val68.

The structures of the H64Q derivatives are similar to those of the H64A mutant in terms of the points of attachment of the aryl ligands to the Fe centers and these ligands eclipsing diagonal porphyrin N atoms. However, although the tolyl ligand plane (Fig. 2C) has a similar tilt (~8°) with respect to the heme normal as seen in the H64A derivative, the chlorophenyl ligand plane (Fig. 2D) has a significantly smaller (~7°) tilt from the heme normal than that observed in the H64A mutant (~18°). The calculated distances between the chlorophenyl ligand atoms (Fig. 2D) and closest distal pocket residues are >3.6 Å.

The X-ray crystal structures in Fig. 2 provide valuable information that can be used to address substituent and distal pocket influences on the ligand conformation in these bioorganometallic Fe–C complexes. A superposition of these structures on those of the respective sw metMb precursors [19,21,22] is shown in Fig. 3. As noted above, both the H64A and H64Q mutants gave the same reaction products upon reactions with the arylhydrazines, both in terms of ligand identity and mode of attachment, with no clear hydrogen-bonding interactions involving the ligands. We focus our discussion on the sidechains of the residues Val68, Gln64, and Phe46, and the CD loop region.

In the H64A–tolyl-*m* structure, the Val68 sidechain flips to a "vertical" orientation from its initial horizontal orientation in the metMb H64A structure, creating a 3.7 Å distance between this sidechain and the centroid of the aryl ring. This Val68 flip relieves a steric clash with the tolyl ligand. In contrast, the Val68 sidechain does not flip in the H64A–chlorophenyl structure, probably due to the significant ~18° tilt of the aryl ligand from the heme normal and away from this residue. Interestingly, this same aryl ligand tilt results in a 45° rotation of the aryl plane of Phe46 and its subsequent movement away from the heme pocket. Within the backbone structure, the largest change occurs in the H64A–chlorophenyl derivative when compared with the metMb precursor and the tolyl complex. In the H64A–chlorophenyl derivative, a ~1.0 Å downward movement of the CD loop region that contains Phe46 is evident (Fig. S5 in the SI), and is likely associated with the change in Phe46 orientation induced by the chlorophenyl tilt in the pocket.

In the related H64Q derivatives, the Val68 sidechains flip to a vertical orientation for both the tolyl and chlorophenyl complexes, when compared with the starting H64Q metMb structure (Fig. 3B). Interestingly, a major orientation change occurs for the Gln64 sidechains in the tolyl and chlorophenyl derivatives from the original position in the H64Q metMb precursor where it H-bonds with the H_2O ligand to Fe. The new Gln64 orientation in the tolyl and chlorophenyl derivatives establishes a new H-bonding contact with Asp60 thus stabilizing this conformation. To the best of our knowledge, this Gln64 orientation shift away from the distal pocket interior towards the exterior is the first such observed movement in any single or multi-mutant H64Q structure reported. Unlike that observed in the H64A structures, no significant movement of the CD loop region is observed in these H64Q structures.

There are several other points to note regarding the crystal structures shown in Figs. 2 and Fig. 3. First, these products resulting from a rather complicated reaction of arylhydrazines with the metMb heme proteins were obtained from soaking pre-formed crystals of the metMb precursors with the arylhydrazine reagents, and show only one preferred conformation for each of the bound aryl ligands. Given that both H64A and H64Q mutants gave the same ligand identities in the products, it seems that the nature of the distal pocket mutation is relatively unimportant in these reactions reported here. The results also suggest that the aryl radical intermediates that form during the heme-induced reaction are generated in close proximity to the Fe centers to enable rapid attachment of the ligands to the Fe, resulting in a single attachment isomer from the arylhydrazine precursors. Consistent with previous NMR studies by others on wt sw Mb–aryls [9,17], we do not observe any migrations of the aryl ligands to heme pyrrole N-atoms. The observed distal pocket changes suggest a role for Val68 in that it may flip its orientation as a function of bound ligand to accommodate varied sizes and/or orientations of the ligands. Further, larger aryl ligands can be accommodated with additional changes in other distal pocket residues such as Phe46 with concomitant movements in the CD loop region. Notably, the distal pocket Gln sidechains in the H64Q mutant products display significant movement towards the protein exterior to accommodate the new ligands.

[1] To obtain the crystal structures of the Mb-aryl products, crystals of the respective metMb precursor [19] were placed in a droplet (4 μL) on a cover slide that contained a solution of the arylhydrazine (0.0125 M arylhydrazine dissolved in 100 mM Tris•HCl, 1 mM EDTA, pH 7.4 or 9.0, and 3.0 M ammonium sulfate). The cover slide was flipped over and placed on top of a well (in a 24-well format) and sealed with grease; the well contained 100 mM Tris•HCl, 1 mM EDTA, pH 7.4 or 9.0, and 3.0 M ammonium sulfate. This allowed the metMb crystals in the drop to react with the arylhydrazines for 1–14 days at room temperature without drying out. During this process, the cover slides were opened every 2–3 days, and the reaction solutions were exchanged with fresh aerated arylhydrazine solutions (and fresh air introduced into the well volume) in order to ensure completion of the reactions. The product crystals were harvested by cryo-loops and washed with cryosolution (100 mM Tris•HCl, 1 mM EDTA, pH 7.4 or 9.0, 3 M ammonium sulfate, 10% glycerol) and stored in liquid nitrogen. The structures reported here were obtained using the following conditions: (i) H64A at pH 9; ligand soaking times of 2 days for the *m*-tolyl derivative and 14 days for the *p*-chlorophenyl derivative, (ii) H64Q at pH 7.4; ligand soakings of 2 days for the *m*-tolyl derivative and 3 days for the *p*-chlorophenyl derivative.

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