Nitrosoamphetamine binding to myoglobin and hemoglobin: Crystal structure of the H64A myoglobin-nitrosoamphetamine adduct

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ABSTRACT: *N*-hydroxyamphetamine (AmphNHOH) is an oxidative metabolite of amphetamine and methamphetamine. It is known to form inhibitory complexes upon binding to heme proteins. However, its interactions with myoglobin (Mb) and hemoglobin (Hb) have not been reported. We demonstrate that the reactions of AmphNHOH with ferric Mb and Hb generate the respective heme-nitrosoamphetamine derivatives characterized by UV-vis spectroscopy. We have determined the X-ray crystal structure of the H64A Mb-nitrosoamphetamine complex to 1.73 Å resolution. The structure reveals the *N*-binding of the nitroso-*d*-amphetamine isomer, with no significant H-bonding interactions between the ligand and the distal pocket amino acid residues.

Keywords: phenylhydroxylamine, nitrosoamphetamine, amphetamine, myoglobin, hemoglobin, X-ray structure

Abbreviations: Amph, amphetamine; AmphNO, nitrosoamphetamine; AmphNHOH, *N*-hydroxyamphetamine; Mb, myoglobin; Hb, hemoglobin.

1. Introduction

Some commonly prescribed medications contain amphetamine or methamphetamine or a form of either. For example, Adderall®, a drug used to treat attention deficit hyperactivity disorder (ADHD) and narcolepsy, is a combination of amphetamine (Amph; left of Fig. 1) and dextroamphetamine (*d*-Amph). Desoxyn® is a prescription methamphetamine (methAmph) for the treatment of obesity and ADHD. Despite their high medicinal value, Amph derivatives are known to have high potential for abuse [1]. Because of this, amphetamine and methamphetamine are classified by the U.S. Drug Enforcement Agency as Schedule II substances [1].

Figure 1. Chemical structures of amphetamine (Amph), *N*-hydroxyamphetamine (AmphNHOH) and *N*-nitrosoamphetamine (AmphNO).

Interactions of Amph and methAmph with heme proteins are an important component of their metabolism. For example, Amph is oxidatively metabolized by cytochrome P450 to *N*-hydroxyamphetamine (AmphNHOH; middle of Fig. 1) and other products [2]. In addition, in humans, methAmph is oxidatively metabolized by cytochrome P450 via aromatic hydroxylation and/or *N*-demethylation to generate Amph and AmphNHOH [3]. It has been known for decades that both Amph and AmphNHOH react with cytochrome P450 under oxidative metabolism conditions to form inhibitory "455 nm" complexes that are stable in the presence of dithionite but are destroyed by ferricyanide [4-8]. These "455 nm" complexes have been characterized as the nitrosoamphetamine (right of Fig. 1) derivatives containing the active site Fe-AmphNO moiety [7, 9],

however no three-dimensional structural information is currently available for these derivatives. We note that such inhibitory Fe-AmphNO derivatives have also been reported for inducible nitric oxide synthase [10], prostaglandin H synthase [11], and microperoxidase-8 [12, 13], but with no structural characterization of the nitroso binding mode. It is intriguing to note that the oxidative metabolism of Amph to AmphNHOH is not limited to cytochrome P450 activity, as human flavin-containing monooxygenase form 3 (FMO3) has been shown to perform this oxidative metabolism as well [14]. Interestingly, FMO3 has also been located in the human brain where it is hypothesized to perform these same functions [15, 16].

AmphNHOH has been isolated as a metabolic intermediate of Amph in animals [17] and surprisingly, formation of heme-AmphNO derivatives have not yet been reported for the muscle protein myoglobin (Mb) and the blood protein hemoglobin (Hb). It has been suggested previously [12, 13] that Mb- and Hb-AmphNO derivatives are unlikely to form due to the large size of AmphNO and the smaller-size heme distal pocket cavity in these proteins.

In this paper, we report the formation of ferrous Mb-AmphNO and Hb-AmphNO derivatives generated from the reactions of AmphNHOH with the ferric heme precursors. Further, we report the 1.73 Å resolution X-ray crystal structure of one such derivative that definitely establishes the coordination mode of the AmphNO ligand to the heme center. The latter structure also represents the first structural evidence for AmphNO binding to any heme protein.

2. Materials and Methods

2.1. Expression and purification of sw Mbs, and isolation of human Hb

The recombinant wt Mb plasmid was a kind gift from Dr. Mario Rivera (Univ. of Kansas). The wt and H64A mutant were expressed in *E. coli* BL21(DE3) and purified as described by Springer and Sligar [18].

Human blood was obtained from the Blood Bank at the University of Oklahoma Health Science Center in the form of packed cells. OxyHb was isolated from the packed cells and purified following published methods [19, 20]. OxyHb was oxidized to metHb by adding 3 to 5-fold potassium ferricyanide as reported previously [19].

2.2. Crystallization of the sw metMb H64A mutant

Crystals of the recombinant H64A metMb protein were grown as described by Phillips et al [21, 22]. The batch method was applied for crystallization, in which 10-20 μ L of 60 mg/ml protein was mixed with 3.2 M ammonium sulfate in 100 mM Tris•HCl, 1 mM EDTA, pH 9.0 to reach 2.3-2.6 M ammonium sulfate as the final concentration.

2.3. Reactions of the heme proteins with N-hydroxyamphetamine

N-hydroxyamphetamine was synthesized following published protocol [23]. The purity was checked by ^1H NMR spectroscopy, and the final product was dissolved in methanol for use in the UV-vis studies.

UV-vis spectroscopy was used to monitor the room temperature (22 ± 1 °C) aerobic reactions of the heme proteins with N-hydroxyamphetamine. To establish the starting point of the reaction involving metMb, 10 μ L of the protein (40 mg/mL) was added to 2.5 mL of 0.1 M sodium phosphate buffer at pH 7.4 in a quartz cuvette (Starna cells). 10 μ L of N-hydroxyamphetamine (90 mM) was then added to the solution. Readings were taken at different time points along the course of the reaction to monitor its progress. Similar procedures were used for the other heme proteins as displayed in the figure captions. The extents of formation of (i.e.,

conversion to) the products were determined by spectral deconvolution using the program OriginPro (OriginLab, Northampton, MA).

To prepare the H64A Mb derivative in crystalline form, appropriately sized crystals of sw H64A metMb were transferred into a 4 μ L droplet (well solution containing 10% glycerol) on a cover slide. The cover slide with the drop was placed in a layer of light mineral oil. A few grains of solid *N*-hydroxyamphetamine were added into the droplet and the color slowly changed from red to pink signifying the formation of Mb H64A-AmphNO. After a soaking time of 2 d, the treated crystals were harvested and stored in liquid nitrogen until X-ray diffraction data collection.

2.4. X-ray data collection

The diffraction data were collected at our home source using a Rigaku RU-H3R X-ray generator coupled to a R-Axis IV⁺⁺ detector. The data were collected at 100 K with CuK α radiation ($\lambda = 1.54178$ Å) from the generator operated at 50 kV/100 mA. 1° oscillation angles were collected over a 100° range with an exposure time of 5 min per image and a crystal-to-detector distance of 110 mm.

2.5 Data processing, structure solution and refinement

The data were processed using *iMosfilm* and *SCALA* as implemented in the CCP4 program suite [24]. The structure factors were calculated using the CCP4 program suite [25]. The phase problem was solved by molecular replacement using *PHASER* (CCP4) [26]. The starting model used was wt swMb (metMb-H₂O) at 1.5 Å resolution (PDB accession code: 2MBW) with the heme, water molecules, and ligands removed from the structure. All refinements were per-

formed using *Refmac5* (CCP4) [27, 28]. Models were rebuilt using *COOT* [29]. *MolProbity* was used for structure validation to check for unusual residue conformations contacts [30].

Ten initial cycles of restrained refinement were run with *Refmac5*, and the *R* factor decreased from 0.355 to 0.275. Ligands (AmphNO) and water were added to the model based on the F_o-F_c electron density maps in the subsequent refinement cycles. The model of AmphNO comes from the X-ray crystal structure of the heme model compound (OEP)Fe(ONCH(CH₃)CH₂Ph)(1-MeIm)) (unpublished; OEP = dianion of octaethylporphyrin). To perform restrained refinement in *Refmac5*, the atoms of AmphNO were selected from this compound in *Pymol* and saved as another pdb file which was input into *Prodrgrg* (CCP4 suite) to generate the coordinates in a new pdb file and the topologies in a monomer library cif file. Two conformations for each of the sidechains of Lys87, Lys133, Lys145 and Gly153 were modeled with 50% occupancy each. The N-terminal Met residue was omitted because of the lack of electron density. The final *R* factor and R_{free} were 0.149 and 0.177, respectively.

The figures were generated using *Pymol* [31]. $2F_o-F_c$ electron density maps (e.g., Fig. S6) were initially calculated by Fast Fourier Transform (*FFT*) in the CCP4 software package [32]. The resulting *map* files were converted to CCP4 files and displayed in *Pymol*. In general, each F_o-F_c electron density map was generated as follows: (i) a new PDB file in which ligands were removed from the pocket was initially refined for 5 cycles in *Refmac5*; (ii) the new *mtz* file that was generated from the first step was input into *FFT* to generate the *map* that was then displayed in *Pymol*.

3. Results and Discussion

Addition of excess *N*-hydroxyamphetamine to an aerobic solution of ferric wild-type sperm whale Mb (wt sw metMb) in phosphate buffer at pH 7.4 resulted in spectral changes in the UV-vis spectrum (Fig. 2A). The Soret band of the precursor wt metMb shifted slowly from λ_{max} 409 nm to 423 nm over a 24 hr period (Fig. S1A), and new bands at 543 and 576 nm were also evident. These new spectral bands are indicative of slow (~22%; Fig. S2) conversion of the metMb precursor to the ferrous Mb-*nitrosoalkane* derivative over this period; other Mb(RNO) complexes display absorption bands in these regions [2, 33]. Similar UV-vis spectral shifts were observed when excess *N*-hydroxyamphetamine was added to an aerobic solution of the metMb H64A mutant in phosphate buffer at pH 7.4; the Soret band shifted from 408 nm to 426 nm, and new bands at 541 and 573 nm were evident (Fig. S3). In the case of this H64A mutant, a ~66% conversion to the ferrous Mb-*nitrosoalkane* derivative was observed after 40 min (Figs. S1B and S4).

Figure 2. UV-vis spectral changes during the aerobic reactions of wt sw metMb (A) and metHb (B) with *N*-hydroxyamphetamine. Conditions: 0.1 M phosphate buffer, pH 7.4, [Mb] = 2.0–2.5 μM or [Hb] = 12.5 μM , final [reagent] = 80–100 μM .

The aerobic reaction of excess *N*-hydroxyamphetamine with human tetrameric metHb in phosphate buffer at pH 7.4 results in analogous spectral shifts as shown in Fig. 2b; the Soret

band shifts from 406 nm to 421 nm, and new bands at 541 and 559 nm appear, with a ~77% conversion occurring over a 4 h period (Figs. S5 and S6).

Once formed, these ferrous nitrosoalkane derivatives are fairly stable in aerobic solution, showing only mild decomposition over time for the Mb products (~12% decomposition over 7 d for wt Mb, ~14% decomposition over 16 d for H64A Mb) and no visible decomposition for the Hb derivative even after 3 d as judged by UV-vis spectroscopy. That these derivatives are in the ferrous form is evidenced by the fact that ferricyanide oxidation converts them back to their respective ferric precursors (e.g., Fig. S7).

To properly characterize the identity of the ferrous products, we attempted to obtain X-ray diffraction quality crystals for all three derivatives. We have, to date, not been successful at obtaining crystals of any of these products via the co-crystallization method (i.e., crystallization from solution after the product is formed). However, we were successful at obtaining crystals of only the ferrous H64A derivative via the crystal soaking method, where *N*-hydroxyamphetamine is added to crystals of the H64A metMb precursor (Fig. S8). Specifically, a few grains of solid *N*-hydroxyamphetamine were added to crystals of H64A metMb in aerobic buffer (0.1 M Tris•HCl, 1 mM EDTA, pH 9.0, 2.3 mM (NH₄)₂SO₄). The crystals changed color from red to pink over a 1 d soaking period. We found, however, that the best condition to obtain diffraction quality crystals of the product was if the soaking was performed over 2 d.

The crystal structure of the ferrous H64A Mb product in the *P*6 space group was obtained at 1.73 Å resolution (Table S1). The heme active site of this H64A Mb-nitrosoalkane derivative is shown in Fig. 3, and the $F_o - F_c$ omit electron density map shown in the green mesh clearly reveals the presence of the AmphNO ligand (Fig. 1, right) bound to heme Fe.

There are several points to note regarding this H64A Mb-AmphNO structure: (i) the nitrosoamphetamine ligand is bound to heme Fe through its nitroso N atom, (ii) only one isomer, namely the *d*-Amph isomer, is bound to the Fe center, (iii) the Fe-N-O moiety ($\text{FeNO} = 108^\circ$) is

Figure 3. The $F_o - F_c$ omit electron density map (green mesh, contoured at 3σ) and final model of the heme active site of the ferrous sw H64A Mb-nitrosoamphetamine derivative (PDB accession code 5KD1). The $2F_o - F_c$ map is shown in Fig. S9. In this view, the Thr67 and Val68 residues are located behind the nitrosoamphetamine ligand and do not contact it.

perpendicular to the heme plane and essentially eclipses a heme pyrrole N atom, and (iv) there are no H-bonding interactions between the nitrosoamphetamine ligand and the distal pocket residues. Conformation changes in distal pocket amino acid side-chains when compared with the metMb H64A precursor (rmsd of 0.27Å for main chain $\text{C}\alpha$ atoms, and 0.67Å for all atoms) are shown in Fig. S10; the Val68 side-chain flips to a "vertical" orientation from its original horizontal position in the metMb H64A precursor, and the $\text{C}\alpha$ atom of the Phe46 residue shifts by 1.5Å to accommodate the ligand.

The crystal structure of the H64A Mb-AmphNO derivative, and the similarity of its UV-vis spectrum with those of wt Mb and Hb suggest that similar *N*-binding modes are extant in both Mb and Hb. Importantly, our results show for the first time that bulky amphetamine metab-

olites such as the nitroso derivative can bind to the relatively small heme sites both Mb and Hb. The formation of heme-AmphNO complexes with Mb and Hb from AmphNHOH suggests that the interactions of AmphNHOH/AmphNO with heme proteins may not be limited to those with large active site pockets such as the cytochrome P450s.

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Supporting Information: Additional UV-vis spectra; crystallographic data and additional figures (PDF)

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. BW (protein expression, crystallization, crystal soaking, structure solution/refinement, UV-vis), SMP (wtMb /Hb UV-vis), GY (ligand synthesis,

H64A UV-vis, crystal soaking), NX (ligand synthesis), GBRA (project coordination). Taken in part from the Ph.D. thesis of GY.

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