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Laser flash photochemical oxidation to locate heme binding and conformational changes in myoglobin

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Abstract

Fast photochemical oxidation of proteins (FPOP), a means of protein footprinting, uses OH radicals to oxidize the solvent-exposed residues of proteins on a short time scale (~1 μs). A 248 nm pulsed laser beam dissociates H_2O_2 (15 mM) into hydroxyl radicals. The radicals react with exposed and reactive amino acid residues (e.g., C, M, W, Y, F, H, L, I) in competition with quenching with a glutamine scavenger present in solution. We report here the use of FPOP to confirm that the F-helix is conformationally constrained in the holo form of myoglobin, whereas it is conformationally free in the apo form. The interpretation finds support in the differences in oxidation of various residues in the apo versus holo forms. The differential reactivity of leucine 137 suggests that it is part of a hinge region on the H-helix, enabling the binding pocket to close in the apo form. This is our second study that offers support that FPOP is capable of elucidating the conformational dynamics of proteins by obtaining information that may not be accessible by other methods.

Keywords: Fast photochemical oxidation of proteins (FPOP); Hydroxyl radical footprinting; Holomyoglobin; Electrospray ionization; Protein conformation

1. Introduction

There have been remarkable advances in the ability of mass spectrometry to measure the binding affinity and determine the site of binding in proteins. Numerous mass spectrometry-based methods, beginning with the detection of protein/ligand complexes, can determine the affinity and the binding location of a ligand in a complex [1–10]. Given that mass spectrometry measures the mass-to-charge ratio of an analyte, it is necessary that a mass change occur that is proportional to the protein–ligand interaction. Thus, nearly all mass spectrometric methods require that a change in reactivity of the protein or the ligand occurs between the bound and unbound forms.

A number of approaches use photoaffinity labeling [11–13] and covalent modification of the protein at sites except those involved in ligand binding. These approaches are examples of a general strategy called "chemical footprinting," which uses chemical reactions of either an amino acid side chain or an amide proton [14–16] to assess binding. Among these methods, hydrogen/deuterium amide exchange has attracted the widest interest. Its origins for understanding protein structure are in the 1950s [17,18], and it has been widely used by NMR spectroscopists since the 1980s [19]. Most of the effort since the 1990s, when an explosive increase in the use of H/D exchange for biophysical characterization occurred, has been with NMR, however, not with mass spectrometry [20].

Advantageously, H/D exchange by mass spectrometry can determine both the binding affinity [21–23] and the location of the binding interface [16,24–27]. Although these determinations require separate experiments, the chief advantages of mass spectrometry are its high sensitivity and speed. The use of the pepsin protease to digest the exchanged protein under "quench" conditions at low pH improves the resolution to the peptide level. Efforts to increase the resolution further to the amino acid level by using collision-induced dissociation may bear fruit, but there is concern because deuterium on protected amides may be randomized during in vacuo (mass spectrometric) sequencing processes [28–30]. Although mass spectrometry methods have speed and sensitivity advantages compared to NMR spectroscopy, NMR offers residue-specific information and real-time monitoring of the back-exchange process, whereas mass spectrometric methods, which do not

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allow an in situ analysis, must quench the exchange under conditions of minimal back-exchange and then measure the extent of deuteration at a particular point in time.

Protein footprinting in which an irreversible chemical change is introduced (e.g., acetylation of lysine) are not susceptible to back-exchange problems. Many of these approaches suffer from lack of reactivity of most amino-acid side chains. A species that reacts with a reasonable number of amino acid side chains is the hydroxyl radical, and it is a good probe for macromolecular binding interactions [31–35]. The high reactivity is advantageous because the modifying reactions are completed on a short time scale. For example, the amino acids, cysteine, tryptophan, and tyrosine, have diffusion-limited reactivity, whereas phenylalanine, methionine and histidine are only slightly less reactive [36]. Highly exposed leucine and isoleucine residues are also reactive, expanding the generality of the modification scheme [37]. For most of these residues, the oxidative products cause a mass shift of +16 Da, but tryptophan and histidine undergo various oxidation reactions that lead to alternate end-products [38]. In total, eight (C, W, Y, F, M, H, L, I) of 20 possible amino acid residues can be readily labeled by reacting with $^{\bullet}$OH, although another seven have been tested in model systems [38]. Once modified, sites of oxidation can be located by using digestion and MS/MS methods (standard analytical proteomics) provided that the peptide fragmentation pattern leads to unequivocal identification of the exposed residue [37,39].

Chance and co-workers [40], who use synchrotron radiation to generate OH radicals by ionization of water solvent molecules, have been the strongest advocates of using OH radical foot printing in protein studies. As part of their protocol, they measure first order kinetics for disappearance of the unmodified protein to obtain quantitative data on exposure of a residue. Moreover, single phase kinetics is a criterion for "single-hit" conditions. This is important because radicals can inactivate proteins by causing unfolding, leading to an increase in hydrophobicity and exposure of residues that would not be reactive in the wild-type protein, [41,42], and to degradation [43–45]. Under "single-hit" conditions, only the folded form of the protein is oxidized, insuring that the oxidation sites are those of the wild-type protein conformation. Another means of insuring that oxidation does not provide misleading data is to accomplish the oxidation so rapidly that the protein cannot unfold during the time of reaction. This is the motivation for fast photochemical oxidation of proteins for footprinting (FPOP).

Given the success of the radical footprinting method, as demonstrated by Chance, there is a motivation to develop other more readily available means for generating radicals [46]. The use of the Fenton reagent [46,47] and photochemical oxidation at 5 M H_2O_2 [48,49] are two examples. FPOP, developed in our laboratory [37] and that of Aye et al. [50], is a new method that uses photochemical oxidation but under drastically different conditions. In our approach, radicals are formed by homolytic cleavage of 15 mM hydrogen peroxide by absorbing a 17 ns pulse of 248 nm light from a KrF excimer laser. The solution contains, in addition to the protein, 15 mM glutamine, which quenches the radicals in approximately 1 μs. There is significant evidence that indicates that even super-secondary structure packing cannot unfold on this time scale [37,51–55].

Using this approach, we demonstrated previously that the apo form of myoglobin has a conformationally flexible F-helix, in accord with NMR evidence [56], and is undergoing the conformational changes on the sub-millisecond timescale. Limited proteolysis of the F-helix is also proof of its solvent exposure [57]. FPOP also demonstrated that the heme-binding pocket must be closed on the microsecond timescale. Given that the pocket is lined with hydrophobic residues, primarily leucine and isoleucine, this result is not surprising, but no other study of apo myoglobin had previously revealed this phenomenon. The purpose of the work reported in this article is to investigate whether FPOP can locate the heme-binding pocket and detect differences in the conformational changes in myoglobin between the apo and holo forms.

2. Methods

All chemicals including the proteins were obtained from Sigma Aldrich Chemical Company (St. Louis, MO). A 248 nm KrF excimer laser (GAM Laser Inc. Orlando, Fl) with 50 mJ per pulse output, combined with an external pulse generator (B&K Precision, Yorba Linda, CA) was used to photolyze H_2O_2 in all experiments. The laser was focused through a 250-mm convex lens (Edmunds Optics, Barrington, NJ) onto 150-μm i.d. fused silica tubing (Polymicro Technologies, Pheonix, AZ) located 125 mm from the lens. To form a UV transparent window, the polyimide coating over a length of 2.5 cm was removed with a propane torch.

To label surface accessible residues, a solution with a final concentration of 10 μM holomyolgobin, 10 mM NaH_2PO_4 (pH 7.8), 150 mM NaCl, 15 mM glutamine and 15 mM H_2O_2 was used (∼0.05% H_2O_2). The hydrogen peroxide was added from a 150 mM H_2O_2 stock solution just prior to oxidation. The sample (in 50 μL of solution) was loaded into a 100 μL gas-tight syringe and introduced via a syringe pump coupled to the fused silica tubing. The flow rate was set to 20 μL/min, and the laser frequency to 7 Hz. This guaranteed that each bolus of protein was oxidized only once, as described previously [37]. The capillary outflow corresponding to ∼1000 laser shots over 2.5 min was collected in a microcentrifuge tube, to which was immediately added 1 μL of 1 μM catalase to remove any remaining H_2O_2.

To measure the extent of oxidation, the usual procedure was to load 5 μL (containing 50 pmol total protein) of the collected sample onto a C_{18} Opti-guard column from Optimize Technologies (Oregon City, OR) that had been pre-equilibrated with 150 μL of water. The sample was then desalted with 350 μL of water. The protein was eluted using 20 μL/min 50% CH_3CN in 0.1% formic acid into the mass spectrometer. The mass spectrometer was a Waters Ultima Global Quadrupole time-of-flight (Milford, MA), operating in the W mode at ∼15,000 mass resolving power. The summed data were deconvoluted with MaxEnt 1, supplied by the instrument manufacturer, after selecting appropriate mass ranges, a "resolution" of 0.5 and a Gaussian

damage model of 0.025 to model the isotopic envelope of the protein.

The remaining protein was then digested with trypsin at a 1:20 (w/w) ratio of enzyme:protein. Following 24-h incubation at 37 °C, the peptides were desalted using a C18 ZipTip (Millipore, Billerica, MA). The resulting solution was lyophilized and resuspended in 10 μL of solvent A (97% H_2O, 3% CH_3CN, and 0.1% formic acid (FA)). 1.0 μL of sample was loaded onto a silica capillary column with a PicoFritTM tip (New Objective, Inc., Woburn, MA) that was custom packed with C18 reverse-phase material (Delta-Pak, 0.075 mm × 100 mm, 5 μm, 300 Å, Waters Corp., Milord, MA). The gradient was pumped by using an Eksigent NanoLC-1D (Livermore, CA) and was from 0% solvent B (97% CH_3CN, 3% H_2O, 0.1% FA) to 60% solvent B over 60 min, then to 80% solvent B for 10 min at an eluent flow of 260 nL/min followed by a 10 min re-equilibration step. The flow was directed into the entrance of the heated capillary of an LTQ-FT mass spectrometer (Thermo-Electron, San Jose, CA). A mass spectrum of eluting peptides was obtained at high mass resolving power (∼100,000 for ions of *m*/*z* 400) with the FT mass spectrometer component while MS/MS experiments on the nine most abundant eluting ions were rapidly performed in the LTQ at a collision energy of 33% of the maximum energy available, selecting the precursor mass over a range of 0.5 Da below to 4.0 Da above and using wide-band activation. Ions that were submitted to MS/MS were then placed in a dynamic exclusion list for 30 s.

The data were searched by using Mascot (Matrix Sciences, London, UK) against the MSDB database to identify the unmodified peptides from myoglobin. All unmodified peptides were then manually searched for known oxidations that lead to mass modifications of +15.9949 Da, +31.9898 Da, +4.9789 Da and −22.0320 Da adjusted according to the charge state of the precursor ion. Product-ion spectra of the modified peptides were then manually interpreted by comparing them with the product-ion spectra of the unmodified tryptic peptides, and an assignment was made when there was sufficient certainty that a particular residue had been modified. A custom correlation software and data analysis program (developed by Dr. Ilan Vidavsky in this laboratory) was used for searching the experimental data and locating modified peptides, although selected ion chromatograms were still verified manually to locate all possible modified peptides.

To calculate solvent exposure of all residues, the solvent exposure calculator available on-line at http://molbio.info.nih.gov/structbio/basic.html was used. This calculator, which was implemented by Susan Chacko based on previously published work [58], allowed us to compare readily the apo and holo forms of myoglobin as the calculator was able to account for the porphyrin group in the holo form.

3. Results and discussion

3.1. Experimental results

The goal of this study is to use FPOP to investigate the differences between the apo and holo forms of myoglobin. Given

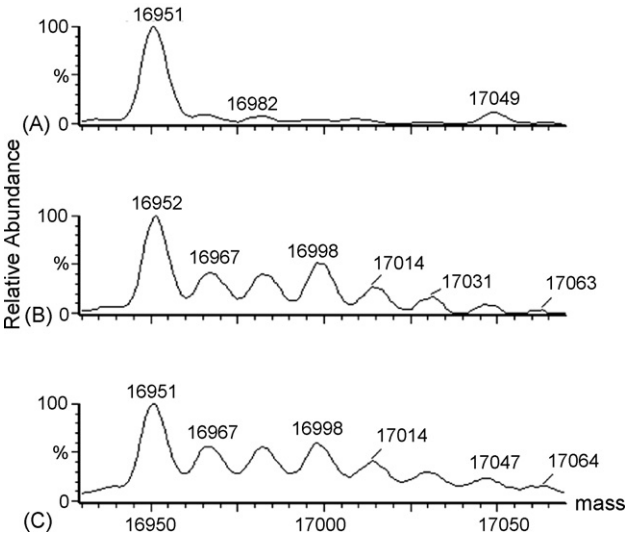

Fig. 1. Treatment of (A) holomyoglobin incubated with 15 mM H_2O_2 for 10 min, (B) holomyoglobin laser irradiated with 15 mM H_2O_2 and 15 mM Gln and (C) apomyoglobin under identical conditions.

that the holo form of myoglobin contains a heme group, we tested whether, in the absence of UV irradiation but under oxidation conditions, the Fe in heme can catalyze oxidation via the Fenton reaction. The data in Fig. 1A show that there is a very small amount of single and double oxidation present as a result of 10 min of incubation with 15 mM H_2O_2 and no scavenger. Therefore, it is not surprising that when we incubate the sample with scavenger for only 3 min, the amount of time it takes to run an FPOP experiment, we found no detectable signal for any oxidized protein (data not shown).

The outcome of oxidation of the holo and apo forms of myoglobin (Fig. 1B and C) is revealed by the two deconvoluted ESI mass spectra that show that the number of oxidations observed for the two forms is nearly identical. A small relative abundance difference may be present; however, this may be due to weak protein signal in the holomyoglobin raw data.

Following the oxidation, we digested the holomyoglobin sample with trypsin and analyzed the mixture of peptides with the combined ion trap-FTMS instrument, enabling the accurate mass and the fragmentation pattern of the peptides to be determined in a data-dependent experiment. The oxidations that we observed (based on a "yes" or "no" format) are listed in Table 1 and are compared to oxidations observed from apomyoglobin [37].

The data presented in Table 1 show that methionine, tryptophan, and tyrosine residues are modified in both the apo and holo forms of the protein. No judgments about peptides containing tryptophan 14 and tyrosine 103 could be made because there was some chymotryptic activity in the trypsin, and the resulting peptides containing these sites are small and hydrophilic. Thus, they did not bind to the HPLC column and were lost in the analysis. The oxidation of phenylalanine reveals solvent exposure, as demonstrated by the results for Phe 43, which is oxidized only in the apo form. In the holo form, Phe 43 loses its solvent exposure because it is part of the ligand-binding pocket and occupies the front edge of the protein where it stabilizes the hydrophobic

Table 1
Observed oxidations on myoglobin

Amino acid		Solvent exposure (Å^2)		Observed oxidation	
Type	Number	Apo	Holo	Apo	Holo
MET	55	16	16	Y	Y
MET	131	0	0	Y	Y
TRP	7	19	19	Y	Y
TRP	14	7	7	Y	nd
TYR	103	28	12	Y	nd
TYR	146	18	18	Y	Y
PHE	43	56	6	Y	N
PHE	46	8	8	N	N
PHE	106	36	36	Y	nd
PHE	123	9	9	N	N
PHE	138	30	0	N	N
PHE	151	38	38	Y	Y
HIS	24	12	12	Y	Y
HIS	36	29	29	Y	Y
HIS	48	61	61	Y	Y
HIS	64	28	12	Y	Y
HIS	81	64	64	Y	Y
HIS	82	9	9	N	N
HIS	93	37	0	Y	N
HIS	97	38	17	nd	nd
HIS	113	48	48	Y	nd
HIS	116	47	47	Y	nd
HIS	119	24	24	Y	Y
ILE	21	54	54	Y	Y
ILE	30	14	14	N	N
ILE	75	14	11	N	N
LEU	72	27	0	N	N
LEU	76	0	0	N	N
LEU	86	10	10	Y	N
LYS	87	58	58	N	Y
PRO	88	46	46	Y	Y
LEU	89	56	22	Y	N
LEU	137	50	50	Y	N
LEU	149	55	55	N	Y

Solvent exposure for the apo form assuming a static structure in the absence of the heme ligand. The annotation Y is used when an oxidation is observed and identified for a particular residue. The annotation N signifies that no oxidation was observed for that residue. The annotation "nd" indicates that no peptide offering sequence coverage for this residue could be seen. This appears only in the holo sample as significant chymotryptic acitivty was observed with this lot of trypsin.

heme group. Other phenylalanine residues (e.g., Phe 151) in the protein are significantly solvent-exposed and are oxidized, as expected, whereas Phe 46 and 123, which have less than 10 Å^2 of solvent exposure, are not modified.

This is consistent with private communications that indicate that highly reactive residues with 10 Å^2 of solvent exposure can undergo oxidation. To reconcile solvent exposure and chemical reactivity, the Hettich lab [59] introduced the concept of reactivity × solvent exposure as a reliable predictor of oxidation between all amino acid residues. For example, leucine, a less reactive residue, needs to be more solvent-exposed for oxidation to occur than phenylalanine, a residue that is approximately five times more reactive [60].

Phe 138 is not modified in either the apo or holo states. We found previously that there would be 30 Å^2 of solvent exposure in the apo form for Phe 138 if the heme binding pocket remained open (if this were a static protein structure) [37]. The binding pocket, however, is composed of many isoleucine, leucine, and valine residues as well as Phe 138, which is located at the back of the binding pocket. Given that no detectable oxidations occur in the binding pocket, in particular at the highly reactive Phe 138, we proposed that the binding pocket closes in the absence of heme ligand. The proposed conformational change would exclude water from the binding pocket, presumably increasing the entropy of the system relative to an open binding pocket where water molecules must organize to hydrate the pocket. In the holomyoglobin form, the heme fills the pocket, excluding water from the hydrophobic pocket (protecting Phe 138 with 0 Å^2 of solvent exposure).

The results for oxidation of leucine 137 corroborate this interpretation. In the apo form, peptides with *m*/*z*'s corresponding to the $[M+H]^+$ of oxidized peptides of ALELFR elute at 28 and 36 min (Fig. 2B). The MS/MS sequencing results of both species demonstrate that only leucine 137 is oxidized, presumably at different side-chain positions, but the total amount of oxidation is approximately 3% RA with respect to the unmodified peptide (area representing oxidized peptide/total area under the curve). In the holo form, however, there is no oxidation, as

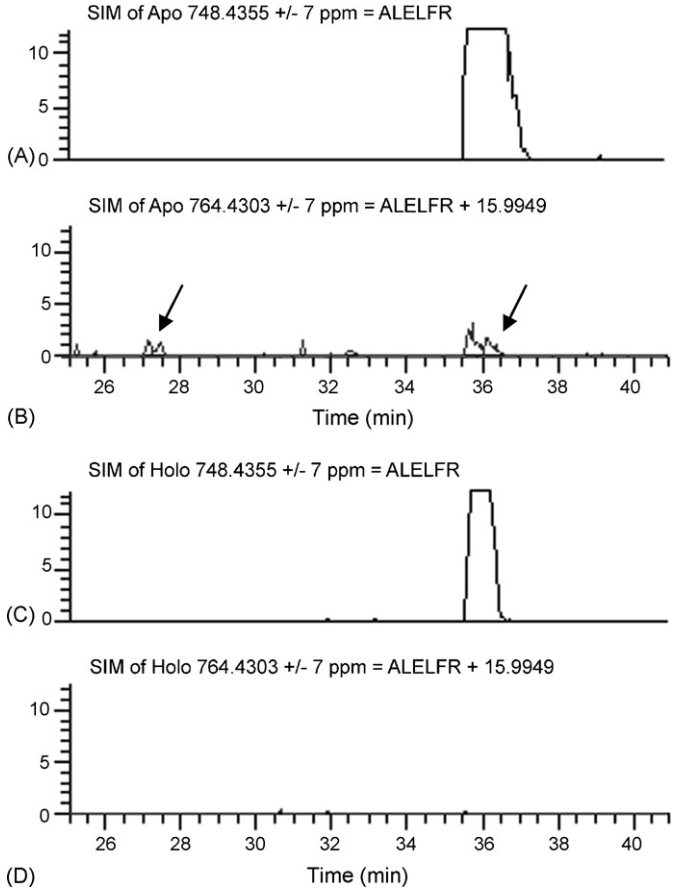

Fig. 2. Selected ion monitoring of the unmodified peptide ALELFR from apomyoglobin (A) and holomyoglobin (C) compared to the oxidized peptide of ALELFR from the apo (B) and holo form (D) of myoglobin. The *Y*-axis has been scaled to 12.5% of the signal intensity of the base peak of the unmodified peptide to magnify the oxidized peptide. The *X*-axis of apomyoglobin has been corrected for chromatography differences.

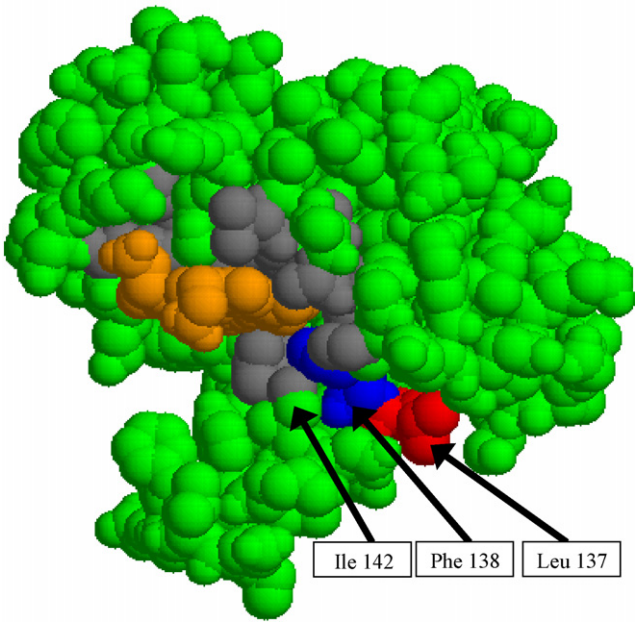

Fig. 3. A space filling model of holomyoglobin with the F-helix removed to simulate conformational dynamics. The heme group is colored in gold, Phe 138 in blue, and Leu 137 in red.

shown in Fig. 2D. The solvent exposure of 50 Å^2 is calculated from the holomyoglobin crystal structure. The absence of oxidation of this residue in the holo form indicates that the residue must be less solvent-exposed in the holo form. The solvent exposure × reactivity method indicates that 50 Å^2 of solvent exposure is the minimum necessary for leucine/isoleucine oxidation.

3.2. Structural analysis of oxidation results

Fig. 3 shows a space-filling model of holo myoglobin (pdb: 1WLA) with the F-helix removed to mimic the van der Waals surface in the apo form of myoglobin. In the apo form, the F-helix is known to be conformationally dynamic [56], and we observe oxidation of Leu 137 in the apo form because it is more exposed compared to the holo form. Considering that our results indicate that the Phe 138 is not solvent-exposed, we propose that the H-helix moves like a lever in the apo form to close the pocket, burying Phe 138, and increasing the solvent exposure of leucine 137.

NMR data corroborate this proposal because the Cα chemical shift index data indicate that the C-terminus of the protein, from residue 143 to 153, has lost α-helical secondary structure in apomyoglobin. Why does residue 142 retain α-helical structure? We propose that it is because the H-helix can move like a lever, maintaining α-helicity, shifting Ile 142 into the binding pocket, burying Ile 142, and Phe 138, but increasing the solvent exposure of Leu 137.

Histidine oxidation is also highly dependent on solvent exposure. Numerous histidine residues with >11 Å^2 of solvent exposure are modified, whereas His 82 (9 Å^2) and His 93 (for holo, the exposure = 0 Å^2) are not modified. His 93 is oxidized in the apo form where it has at least 37 Å^2 of solvent exposure (more if the dynamic nature of the F-helix were taken into account). This large difference is due to the function of His 93, which ligates the heme iron atom in the holo form. The non-covalent bond between His and the heme iron atom stabilizes the F-helix in holomyoglobin, burying His 93 and preventing oxidation. Therefore, the oxidation at this site in the apo form confirms that His 93 is solvent-exposed in apo form, and buried in holomyoglobin.

His 93 is not the only probe for the state of the F-helix. Oxidation data for Leu 86, Lys 87, Pro 88 and Leu 89 of the F-helix (Table 1) show that in the apo form, only the Lys 87 remains unmodified. The abundance of other nearby modification sites may make the Lys modification kinetically non-competitive with respect to the exposed leucine residues proximal to the lysine. This is an example of nearby environment effects on protein oxidation, and these effects cannot always be controlled when proteins are changing conformations. Leucine, which has a rate constant for the amino acid reaction with •OH of $2 \times 10^9\ M^{-1}\ s^{-1}$, is five times more reactive than lysine ($4 \times 10^8\ M^{-1}\ s^{-1}$) [60]; thus, it is reasonable that when oxidation occurs at both exposed leucine residues in the apo form, there is no oxidation at lysine. In the holo form, neither leucine is oxidized whereas both proline and lysine are modified. The differential leucine oxidation confirms the conformational stabilization of the F-helix in the holo form.

Lastly, we observed differential oxidation of Leu 149. In the apo form, the C-terminus has lost secondary structure from residues 143–153, as determined by NMR [56]. Given that Tyr 146 and Phe 151 are 5–10 times more reactive than leucine, we propose that •OH reacts with the two aromatic residues more frequently for the apo form, for which conformational dynamics cause opening of the structure, than for the more rigid holo form, explaining the differential oxidation of Leu 149.

4. Conclusions

FPOP, operating on the microsecond time frame, is capable of differentiating between ligand-bound and free forms of myoglobin. Although the apo form has resisted structural analysis by NMR and X-ray crystallography, we are able to show by FPOP and mass spectrometry that solvent-exposed residues react with hydroxyl radicals, revealing residues that are involved in ligand binding and showing conformational changes that occur as a result of ligand binding. We suggest that the H-helix is involved in closing the hydrophobic ligand binding pocket as evidenced by differential oxidation of leucine 137.

Although it is useful to have the three-dimensional structure of a protein to understand the underlying causes for the differences in the oxidation pattern, we developed FPOP for situations where X-ray data are not available. For example, the Alzheimer Aβ 1–42 peptide is resistant to NMR and crystallographic analysis [61], although a recent 3D model was proposed for the protofibril structure [62]. FPOP footprinting may be applicable to determine additional structural constraints by comparing the monomer to the protofibril. One should not forget that the X-ray structure is of the protein in the solid state, but protein dynamics can only be observed in solution, where FPOP is applicable.

The methodology is applicable to low sample quantities and to experiments at low concentrations where, for example, a

monomer protein is more likely to exist. One can compensate for a decreased concentration by pooling the sample after reaction and then concentrating for analysis. We expect that FPOP should be applicable to a broad array of biological problems currently facing the biomedical sciences. FPOP may also be useful in the context of radical-based side chain H/D exchange of Anderson and co-workers [63].

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