Oxidative Modification of Tryptophan 43 in the Heme Vicinity of the F43W/H64L Myoglobin Mutant*

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The F43W/H64L myoglobin mutant was previously constructed to investigate the effects of electron-rich tryptophan residue in the heme vicinity on the catalysis, where we found that Trp-43 in the mutant was oxidatively modified in the reaction with m-chloroperbenzoic acid (mCPBA). To identify the exact structure of the modified trypthphan in this study, the mCPBA-treated F43W/H64L mutant has been digested stepwise with Lys-C achromobacter and trypsin to isolate two forms of the modified trypthphan which have been separated by size exclusion chromatography. The close examinations of the 1H NMR spectra of peptide fragments reveal that two oxidized forms of the modified tryptophan must have 2,6-disubstituted indole substructures. The 13C NMR analysis suggests that one of the modified tryptophan bears a unique hydroxyl group in stead of the NH2 group at the amino-terminal. The results together with mass spectrometry (MS)/MS analysis (30 Da increase in mass of Trp-43) indicate that oxidation products of Trp-43 are 2,6-dihydroy-2,6-dioxoindole and 2,6-dihydro-2-imino-6-oxoindole derivatives. Our finding is the first example of the oxidation of aromatic carbons by the myoglobin mutant system.

Myoglobin (Mb),1 a carrier of molecular oxygen, can perform oxidation reactions in the presence of hydrogen peroxide (H2O2), although the activity is not as great as that of peroxidase (1–3). The accumulated biochemical and biophysical data allow us to utilize Mb as a heme enzyme model system, and various myoglobin mutants have been constructed to elucidate structure-function relationship on the activation of peroxides (3–5). For example, F43H/H64L Mb, one of the distal histidine relocation mutants, exhibits the enhanced reactivity with H2O2 and the longer lifetime of an active intermediate, a ferryl porphyrin radical cation (O=Fe353porphyrin") (6). Therefore as the results, the F43H/H64L mutant is able to catalyze the sulfoxidation and epoxidation reaction at the rate comparable with the values of peroxides.

On the other hand, cytochromes P-450 (P-450) catalyze the hydroxylation of a wide variety of substrates, including hydrocarbons and polycyclic aromatic molecules (7, 8). The variance in reactivity of Mb and P-450 could arise from differences in the active site structure and the arrangement of functional amino acid residues. The crystal structure of P-450cam with d-camphor reveals that the substrate is tightly bound in the hydrophobic heme pocket through hydrogen bonding interaction with the hydroxyl group of Tyr-96 and the carbonyl oxygen of d-camphor (Fig. 1A) (9). The distance between the heme iron and C5 of d-camphor, the hydroxylation site, is 4.2 Å. On the contrary, the active site of myoglobin is exposed to the exterior and does not provide any specific interactions for accommodating a foreign substrate with high affinity (10). Therefore, it will be difficult for a ferryl porphyrin radical cation of Mb to hydroxylate a substrate molecule, which is not bound in an appropriate position nearby the heme. We hypothesize that a ferryl oxygen atom transfer to aliphatic or aromatic molecules by Mb mutants might be possible even without a proximal thiolate ligand if the substrates were fixed nearby the heme iron.

The F43W/H64L Mb mutant, which was previously constructed to investigate effects of an electron-rich tryptaphan residue on the peroxidase activity, appears to be a good model for examining the hypothesis, because aromatic carbon atoms of tryptophan are fixed in the heme vicinity (Fig. 1B) (12). Although the crystal structure of the F43W/H64L Mb is not available at the moment, the calculated model structure suggests that the distances of Fe–C7 and Fe–C6 are 4.9 and 5.5 Å, respectively. The calculated structure of F43W/H64L Mb was obtained by using the Insight II molecular modeling program (Biovem MSI, San Diego, CA). The Trp-43 in the mutant is generated by replacing His-43 of the F43H/H64L Mb (6) with a tryptophan residue and minimizing the energy of the heme pocket. The predicted values are similar to the distance between C5 of d-camphor and iron in P-450cam. Our earlier studies provided preliminary evidence that Trp-43 in F43W/H64L Mb was oxidatively modified in the reaction with m-chloroperbenzoic acid (mCPBA); however, the exact structure of the modified product(s) remains to be elucidated (12). The subject of this study is to identify the oxidized tryptophan residue to determine whether or not the F43W/H64L Mb mutant is capable of performing the oxidation of aromatic molecules.

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† The abbreviations used are: Mb, myoglobin; P-450, cytochromes P-450; mCPBA, m-chloroperbenzoic acid; FPLC, fast protein liquid chromatography; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TSP-d₄, (trimethyl-silyl)propionic-2,2,3,3-d₄.
EXPERIMENTAL PROCEDURES

The Oxidation of Trp-43 and the Isolation of Peptide Fragments Bearing Trp-43—F43W/H64L Mb (0.2 mM) in 50 mM potassium phosphate buffer at pH 7.4 was mixed with 4 equivalents of mCPBA at 4 °C. The modified protein (500 mg) was digested with Lys-C (1/100 w/w) in 100 mM Tris-HCl buffer at pH 9.0 containing 2 M urea, and the mixture was incubated at 25 °C for 24 h. The digestion was stopped by the addition of trifluoroacetic acid (final concentration 1%). The products were analyzed on an Äkta FPLC system (Amersham Pharmacia Biotech) with a Vydac C-18 reverse phase column eluted typically at a flow of 1.5 ml/min with a gradient of solvent A (0.1% trifluoroacetic acid in water) into solvent B (20% acetonitrile and 0.1% trifluoroacetic acid in water) over 200 min. The eluent was monitored either at 280 nm for aromatic residues or 215 nm for amide bonds in peptide fragments. The isolated fragments A and B, which have been identified as modified Trp-43 linked with unmodified Asp-44-Arg-45-Phe-46-Lys-47 (W*DRFK in which the modified tryptophan is designated as W*) (12), were further treated with trypsin (1/100 w/w) in 100 mM Tris-HCl buffer (pH 9.0) at 30 °C for 12 h and analyzed by the Äkta FPLC system. Although A was not cleaved by trypsin, B was digested to afford a peptide fragment B’ (W*DR). In a control experiment, the intact F43W/H64L mutant (250 mg) was treated with Lys-C and trypsin, and the peptide fragment bearing unmodified Trp-43 was isolated by preparative FPLC.

MS/MS Analysis of the Peptide Fragments—The fragments A and B isolated after Lys-C digestion were directly analyzed on a Voyager DESTR (PerSeptive Biosystems) for MALDI-TOF-MS. The spectrometer was calibrated with an angiotensin II (molecular weight 1046.2) calibration. 1 ml of the digested samples containing ~10 pmol/ml were mixed with 1 μl of saturated α-cyano-4-hydroxycinnamic acid in water/acetonitrile (1:1) and applied to the sample plate by the dried-droplet method.

NMR Spectroscopy—1H and 13C NMR spectra of A, B, and an intact peptide fragment (Trp-43-Asp-44-Arg-45) were obtained either on a Unity Inova 600 MHz or on a Unity plus 600 MHz (Varian). 1H and 13C NMR measurements were undertaken in 20 mM potassium phosphate buffer (pH 7.0) in D2O solution at 25 °C. 3-(Trimethylsilyl)propionic-2,2,3,3-d4 (TSP-d4) was used as an internal reference for proton resonances. Complete proton resonance assignments were made using DQF-COSY (13), TOCSY (14, 15), and ROESY (16) experiments. The ROE intensities of A and B were obtained at 500 and 600 ms mixing time, respectively.

FIG. 1. A, crystal structure of P-450cam; B, calculated structure of F43W/H64L Mb.

SCHEME 1

FIG. 2. MS/MS analysis of the intact WDRFK fragment (A) and product A (W*DRFK) performed on MALDI-TOF (B) (PerSeptive Biosystems, Voyager DESTR). The total masses for the intact and modified fragments are 751.5 and 781.5, respectively. Mass units of the major b and y fragment ions are shown. Essentially the same mass pattern is observed for product B.

FIG. 3. 1H NMR spectra of the unmodified WDR fragment and the modified product A (W*DRFK).
The reaction solution was neutralized with 25/HH$_2$O. The proton signals with coupling constants of 2.6-H$_2$O. The close examination of the 1H NMR spectrum of the modified product shows the comparison of the MS/MS spectra of unmodified and modified fragments. The purified intact peptides (WDR) and the modified fragments (W*DR) were analyzed on a Protein Sequencer (Applied Biosystems, model L-8500A) with a post-column method. The product A and B were analyzed on a High Speed Amino Acid Analyzer (Hitachi, model L-8500A) to confirm the modification site of the intact peptides. The fragment (2 nmol) was hydrolyzed by heating in 25 μl of 4 M methanesulfonic acid at 110 °C for 24 h. The reaction solution was neutralized with 25 μl of 3.5 M NaOH. The peptide sample was loaded on a High Speed Amino Acid Analyzer (Hitachi, model L-8500A) with a post-column method.

RESULTS AND DISCUSSION

The oxidative modification of Trp-43 in F43W/H64L Mb has been performed by adding mCPBA in 50 mM potassium phosphate buffer at pH 7.4. The transition from 5- to 6-coordinated ferric high spin state is a good indication of the protein modification (Scheme 1) (17–21). The mCPBA-treated F43W/H64L mutant is digested with Lys-C achromobacter, and two peptide fragments A and B, which consist of Trp-43(modified)-Asp-44-Arg-45-Phe-46-Lys-47 (W*DRFK), are purified by FPLC. The comparison of the MS/MS spectra of unmodified WDRFK fragment and the modified fragment A. The difference in the spectra provides the direct evidence for the modified site to be Trp-43, and the increased mass number of 30 Da could correspond to the addition of two oxygen atoms and loss of two protons. The MS/MS spectrum of the fragment B is identical to that of the fragment A. Prior to the NMR analysis, A and B have been further treated with trypsin to simplify the NMR spectra. The fragment B affords a shorter peptide defined as B’ (W*DR), trypsin does not cleave the carboxyl side of arginine in the fragment A. Therefore, we have performed NMR analysis of the intact WDR fragment and modified fragments A (W*DRFK) and B (W*DR).

Although the 1H NMR spectrum of the intact WDR fragment exhibits signals derived from five tryptophan protons, only three proton signals of the tryptophan appear in the aromatic region for the modified product A (W*DRFK) (Fig. 3, Table I). The close examination of the 1H NMR spectrum of A reveals that one proton signal at 6.60 ppm couples with two different proton signals with coupling constants of J = 8.2, 2.3 Hz. Two other proton signals appear at 6.58 and 7.19 ppm with coupling constants of J = 2.3 and 8.2 Hz, respectively. The only structure that could afford such a coupling pattern is a 2,6-disubstituted indole substructure as shown in Fig. 4; i.e., the resonance at 6.58 ppm, 6.60 ppm, and 7.19 ppm are unambiguously assigned to the protons at C7, C5, and C4, respectively. The coupling between proton signals at C4 and C5 observed in the COSY spectrum also supports the assignment. Furthermore, the correlation of the C4–H with C3–H (4.14 ppm) and C5–H (2.65 ppm) is observed by the ROESY experiments. The combination of the COSY, ROESY, and TOCSY methods allows us to determine the complete assignment for the fragment A as summarized in Table II. Essentially the same assignment can be applied to the interpretation for the NMR spectra of B’. Two doublet signals at 6.36 ppm (J = 2.5 Hz) and 6.91 ppm (J = 8.5 Hz) are assigned to the C7 and C4 protons of the modified Trp-43 in B’, respectively, and the resonance at 6.26 ppm (J = 8.5, 2.5 Hz) is derived from C5-H (Table I). Therefore, the trypsin residue in the fragment B’ (W*DR) also must have the 2,6-disubstituted indole substructure (Fig. 4). The coupling pattern in the 1H NMR spectrum of the fragment B is essentially the same as those observed in A and B’ spectra.

Amino acid sequence and composition analyses provided us with a clue to identify the structural differences in the modified tryptophan of the fragment A and B’. First of all, A does not react with phenylisothiocyanate, and the Edman degradation does not give us any sequence information, while the sequence analysis for B’ shows Trp*-43(not detected)-Asp-44-Arg-45-sequence. Second, amino acid composition analysis of A reveals that the fragment consists of Asp, Arg, Phe, and Lys; however, the trypsin residue is not identified due to its modification. The results imply that the amino terminus of the fragment A is protected, but the residues except for Trp-43 are intact. To clarify the amino-terminal structure, we have measured 13C NMR of A. The spectrum exhibits a unique signal at 71.1 ppm, which could be assigned as a signal from the hydroxylated carbon atom. Since the fragment A does not contain a serine nor a threonine residue, the comparison of chemical shift for the α carbon in tryptophan (56.1 ppm), indole-3-lactic acid (70.6 ppm), and A (71.1 ppm) suggest that the terminal amino group in A is replaced with the hydroxyl moiety. Thus, we conclude at the moment that the indole substructure in the modified tryptophan is 2,6-dihydro-2-imino-6-oxoindole in A (X−O, Y−NH in Fig. 4) and 2,6-dihydro-2,6-dioxoindole in B terminal (X=Y=O). The proposed structures are consistent with the increase in the 30-Da mass unit with respect to the intact tryptophan. Although further studies are required to clarify the mechanism, a cyclic intermediate generated through the reaction of the terminal amine with the carbonyl carbon atom at the C2 position followed by hydrolysis might be involved to afford 2,6-dihydro-2-imino-6-oxoindole substructure without changing the total mass of the peptide (Scheme 2).

It should be noticed that trypsin somehow recognizes the structural difference in the amino-terminal of pentapeptide A (W*DRFK) and prevents hydrolysis of the carboxyl side of arginine to yield A’ (W*DR). More interestingly, the fragment A is found to be produced from B during the incubation with trypsin or Lys-C, while B is stable in aqueous solution in the absence of peptidases.

|      | C1−H | C4−H | C2−H | C5−H | C6−H | C7−H |
|------|------|------|------|------|------|------|
| Intact fragment | 4.35 | 3.46/3.36 | 7.32 | 7.64 | 7.17 | 7.26 | 7.51 |
| Product A | 4.14 | 2.65/2.26 | d, J = 7.0, 7.0 | d, J = 8.0 | dd, J = 7.2, 8.0 | dd, J = 7.2, 8.0 | d, J = 8.0 |
| Product B’ | 4.46 | 2.90/2.83 | dd, J = 7.0, 7.0 | d, J = 8.2, 2.3 | dd, J = 7.0, 7.0 | d, J = 2.3 |

| TABLE I | 1H NMR chemical shifts (δ in ppm) of Trp-43 in the intact WDR fragment, product A (W-DRFK), and product B’ (W-DR) |
|---------|-----------------------------------------------------------------------------------------------------------------|
|         | C−H                                                                                                              |
|         | Intact fragment                                                                                                     |
|         | 4.35                                                                                                              |
|         | 3.46/3.36                                                                                                          |
|         | 7.32                                                                                                              |
|         | Product A                                                                                                          |
|         | 4.14                                                                                                              |
|         | 2.65/2.26                                                                                                          |
|         | d, J = 7.0, 7.0                                                                                                    |
|         | d, J = 8.0                                                                                                         |
|         | dd, J = 7.2, 8.0                                                                                                   |
|         | dd, J = 7.2, 8.0                                                                                                   |
|         | d, J = 8.0                                                                                                         |
|         | Product B’                                                                                                         |
|         | 4.46                                                                                                              |
|         | 2.90/2.83                                                                                                          |
|         | dd, J = 7.0, 7.0                                                                                                   |
|         | d, J = 8.2, 2.3                                                                                                    |
|         | d, J = 8.2, 2.3                                                                                                    |
|         | d, J = 2.3                                                                                                         |

**Fig. 4. The modified tryptophan structure for both A and B’ on the basis of 1H NMR analyses.**
atom transfer to aromatic molecules would be possible by a heme enzyme with a non-thiolate ligand if the substrates were fixed nearby the heme iron. Our results would coincide with recent genetic analysis, suggesting that a hemoenzyme similar to cytochrome c peroxidase with an imidazole as the proximal ligand is involved in biosynthesis of tryptophan tryptophylquinone, a novel cofactor bearing indole 6,7-dione moiety, in methylamine dehydrogenase (11, 23–24). In addition, we have reported herein that a unique amino-oxo exchange reaction of the amino-terminal 2,6-dihydro-2,6-dioxoindole was performed by Lys-C and trypsin.

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### TABLE II

|          | C–H | C–O | C–H | C–O |
|----------|-----|-----|-----|-----|
| Asp in intact fragment | 4.69 | 2.77/2.62 | 1.51 | 3.11 |
| Arg in intact fragment | 4.10 | 1.80/1.65 | 3.02 | 7.29 |
| Asp in product A | 4.28 | 2.84/2.60 | 4.00 | 1.48 |
| Arg in product A | 4.28 | 2.65/2.50 | 4.00 | 1.48 |
| Phe in product A | 4.61 | 3.26/2.96 | 3.02 | 7.29 |
| Lys in product A | 4.09 | 1.73/1.55 | 1.27 | 1.60 |
| Asp in product B | 4.40 | 2.55/2.45 | 4.15 | 1.79/1.67 |
| Arg in product B | 4.40 | 2.55/2.45 | 4.15 | 1.79/1.67 |
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