Modification of Heme Peptides by Reverse Proteolysis: Spectroscopy of Microperoxidase-10 with C-Terminal Histidine, Tyrosine, and Methionine Residues

Donald W. Low, Grace Yang, Jay R. Winkler,* and Harry B. Gray*

Beckman Institute, California Institute of Technology Pasadena, California 91125

Received January 15, 1997

Small-molecule analogs of the active sites of heme proteins are under investigation in many laboratories.[1−3] Covalent attachment of ligands to the porphyrins is often desirable as it reduces complications due to axial ligand dissociation and scrambling in cases involving mixed axial ligation.[4−9] The preparation of ligand-linked (tailed) porphyrin systems, however, often involves complex multistep syntheses. We have found that trypsin-catalyzed reverse proteolysis[10,11] provides a simple preparative route to a novel class of water-soluble tailed porphyrins based on the well-characterized microperoxidase (MP) framework.[12−23] Using reverse proteolysis reactions, we have obtained mutant microperoxidase decapeptides (MP10s) with C-terminal histidine (H23MP10), tyrosine (Y23MP10), and methionine (M23MP10) residues. In addition, we have investigated the electronic absorption and resonance Raman spectra of these MP10 mutants.

Microperoxidase-9 (MP9), which is a product of trypsin-catalyzed hydrolysis of horse-heart cytochrome *c* (h-cyt *c*), contains residues 14−22 (CAQCHTVEK) covalently attached to the heme by cysteine thioether bonds at positions 14 and 17.[12] The native His18 imidazole axial ligand remains intact in the microperoxidase framework, while the sixth position is occupied by an exogenous ligand.[21,23] Since MP9 is a product of tryptic hydrolysis, we reasoned that it should also readily participate as a substrate in the reverse reaction, trypsin-catalyzed peptide bond formation. Further, the high selectivity of trypsin-catalyzed reactions should minimize side products and circumvent the use of protecting groups. The use of mixed aqueous−organic solvent systems and addition of high concentrations of amino acid nucleophiles shift the peptide bond formation/hydrolysis equilibrium in favor of the peptide synthesis reaction; in fact, we have found that trypsin is effective in promoting peptide bond formation with MP9 as a substrate in 50% aqueous dimethylformamide (DMF) solutions.[24] The product decapeptides were readily separated from the MP9 starting material by reverse-phase FPLC with acetonitrile−water gradients. Due to the high selectivity inherent in trypsin catalysis, only one heme-containing product was observed when enantiomerically pure amino acid amides were employed as nucleophiles. FPLC fractions were assayed by laser desorption mass spectrometery, and fractions containing the desired product were pooled. Reaction yields based on the fraction of MP9 consumed were typically 30−50%.

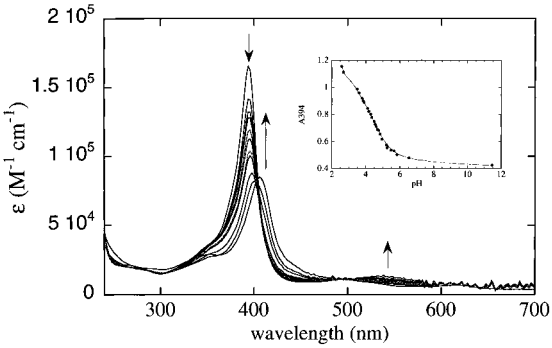

Figure 1. Dependence of the H23MP10 absorption spectrum on pH. The inital pH 1.7 solution was titrated with concentrated NaOH. Spectra were measured with an HP-8452A diode array spectrophotometer. Inset: Titration curve plotting absorbance at 396 nm as a function of pH.

The electronic absorption spectrum of Fe(III) H23MP10 changes dramatically with pH (Figure 1). The green low-pH form has an intense Soret band (λ_{max} 394 nm) that red shifts to 406 nm and loses intensity upon titration with base, giving the high-pH form a deep red color. In the visible region, the low-pH form has broad, relatively weak Q-bands that gain intensity and coalesce as the pH rises. A tight isosbestic point is present at 494 nm, indicating clean conversion (pK 4.4) between the two forms. The spectra indicate that the imidazole of His23 binds axially to Fe(III) at high pH, producing a low-spin heme.[25] The His23 imidazole is protonated at low pH, yielding a high-spin His-aquo heme.[26] A Beer's law plot at pH 7 is linear in the micromolar concentration range (data not shown), suggesting that the imidazole coordination is largely intramolecular in

(1) Tsuchida, E.; Komatsu, T. *Methods Enzymol.* **1994**, *231*, 167−193.
(2) Momenteau, M.; Reed, C. A. *Chem. Rev.* **1994**, *94*, 659−698.
(3) Jameson, G. B.; Ibers, J. A. In *Bioinorganic Chemistry*; Bertini, I., Gray, H. B., Lippard, S. J., Valentine, J. S., Eds.; University Science Books: Mill Valley, 1994; pp 215−246.
(4) Boitrel, B.; Lecas-Nawrocka, A.; Rose, E. *Tetrahedron. Lett.* **1992**, *33*, 227−230.
(5) Battersby, A. R.; Howson, W.; Hamilton, A. D. *J. Chem. Soc., Chem. Commun.* **1982**, *21*, 227−230.
(6) Collman, J. P.; Brauman, J. I.; Iverson, B. L.; Sessler, J. L.; Morris, R. M.; Gibson, Q. H. *J. Am. Chem. Soc.* **1983**, *105*, 3052−3064.
(7) Traylor, T. G.; Berzinis, A. P. *J. Am. Chem. Soc.* **1980**, *102*, 2844−2846.
(8) Traylor, T. G. *Pure Appl. Chem.* **1991**, *63*, 265−274.
(9) Traylor, T. G. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2647.
(10) Nishino, N.; Xu, M.; Mihara, H.; Fujimoto, T. *Chem. Lett.* **1992**, *2*, 327−330.
(11) Bongers, J.; Heimer, E. P. *Peptides* **1993**, *15*, 183−193.
(12) Peterson, J.; Saleem, M. M. M.; Silver, J.; Wilson, M. T. *J. Inorg. Biochem.* **1983**, *19*, 165−178.
(13) Munro, O. Q.; Marques, H. M. *Inorg. Chem.* **1996**, *35*, 3752−3767.
(14) Othman, S.; Lirzin, A. L.; Desbois, A. *Biochemistry* **1993**, *32*, 9781−9791.
(15) Othman, S.; Lirzin, A. L.; Desbois, A. *Biochemistry* **1994**, *33*, 15437−15448.
(16) Wang, J.-S.; Van Wart, H. E. *J. Phys. Chem.* **1989**, *93*, 7925−7931.
(17) Wang, J.-S.; Tsai, A.-L.; Heldt, J.; Palmer, G.; Van Wart, H. E. *J. Biol. Chem.* **1992**, *267*, 15310−15318.
(18) Smith, M.; McLendon, G. *J. Am. Chem. Soc.* **1981**, *103*, 4912−4921.
(19) Satterlee, J. D. *Inorg. Chim. Acta* **1983**, *79*, 195−196.
(20) Huang, Y.-P.; Kassner, R. *J. Am. Chem. Soc.* **1981**, *103*, 4927−4932.
(21) Adams, P. A.; Baldwin, D. A.; Marques, H. M. In *Cytochrome c: a multidisciplinary approach*; Scott, R. A., Mauk, A. G., Eds.; University Science Books: Sausalito, CA, 1996; pp 635−692.
(22) Carraway, A. D.; McCollum, M. G.; Peterson, J. *Inorg. Chem.* **1996**, *35*, 6885−6891.
(23) Low, D. W.; Gray, H. B.; Duus, J. Ø. *J. Am. Chem. Soc.* **1997**, *119*, 1−5.

(24) In a typical reaction, 10 mg of MP9 was added to 1 mL of a 0.5 M solution of the appropriate amino acid amide in anhydrous DMF. Next, 1 mL of buffered solution (pH 7) containing 10−20 mg of DPCC treated trypsin (Sigma) was added to the MP9 solution, resulting in a deep red reaction mixture. After being stirred overnight, the reaction was quenched by addition of excess acetic acid. Organic solvents were removed by washing with 0.1% trifluoroacetic acid in a stirred ultrafiltration cell (Amicon YM-1) before purification by FPLC (Pharmacia PepRPC).

(25) The optical spectrum of the pH 7 form of H23MP10 (λ_{max} = 406, 538 nm) is similar to those of the low-spin imidazole-bound form of MP8 (λ_{max} = 406; 526 nm, data not shown), the semisynthetic M80H mutant of h-cyt *c* (λ_{max} = 415; 524 nm: Raphael, A. L.; Gray, H. B. *Proteins* **1989**, *6*, 338−340), and cytochrome b_5 (λ_{max} = 413; 526 nm: Ozols, J.; Strittmatter, P. *J. Biol. Chem.* **1964**, *4*, 1018−1023). No X-band EPR signal was detected from a frozen (77 K) solution (pH 7) of H23MP10.

S0002-7863(97)00103-0 CCC: $14.00

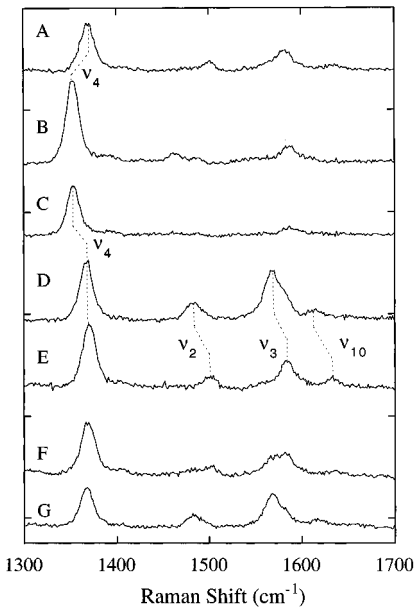

Figure 2. Resonance Raman spectra: (A) Fe(III) Y23MP10, pH 9 0.1 M bicarbonate buffer; (B) Fe(II) Y23MP10, pH 7; (C) Fe(II) H23MP10, pH 7; (D) Fe(III) H23MP10, pH 2; (E) Fe(III) H23MP10, pH 7; (F) Fe(III) MP8, pH 7; (G) Fe(III) MP8, pH 2. Dotted lines between spectra illustrate shifts in marker bands between related forms of MP10. pH 7 solutions were buffered with 0.1 M potassium phosphate. pH 2 solutions were 0.01 M H_2SO_4. Excitation at 406 nm was provided by an Lambda-Physik FL3002 dye laser with use of PBBO dye pumped by an LPX 210i excimer laser operating at 10−50 Hz, approximately 1 mJ per pulse at 406 nm at the sample. The probe light was filtered by using a diffraction grating followed by tight focusing and passage through a 20-μm aperture to reject off-axis light. Raman light was collected with use of a right-angle collection geometry. Scattered light was focused on the aperture of a SPEX 750 M monochromator equipped with a cooled CCD array detector. Typical acquisition times were 1−5 min, with sample concentrations between 50 and 100 μM. All spectra were collected at ambient temperature. Cosmic ray artifacts have been removed from the spectra.

nature. Fe(III) Y23MP10 also undergoes a pH-dependent spin-state change, and its absorption spectrum is characteristic of a low-spin form at pH 9. Fe(III) M23MP10, however, does not exhibit any spin-state changes other than those present in the MP9 precursor, indicating that the thioether side chain of Met23 does not coordinate to the ferric heme center. Interestingly, the thioether of M23MP10 apparently has little affinity for Fe(II); the UV−vis absorption spectrum of Fe(II) M23MP10 is virtually identical with that of reduced MP9.

The resonance Raman spectra of the Fe(III) forms of H23MP10 and Y23MP10 have well-resolved porphyrin marker band regions (Figure 2). The high-frequency bands of H23MP10 (pH 7) (ν_4 = 1368 cm^{-1}, ν_2 = 1584 cm^{-1}, ν_{10} = 1633 cm^{-1}) and Y23MP10 (pH 9) (ν_4 = 1368 cm^{-1}, ν_3 = 1501 cm^{-1}, ν_2 = 1581 cm^{-1}, ν_{10} = 1634 cm^{-1}) resemble those of pure low-spin hemes.[15,27,28] In contrast, the corresponding region of the resonance Raman spectrum of MP8 (pH 7) features broadened marker bands attributable to a heterogeneous mixture of heme chromophores with high- and low-spin states.[16,17] As the pH is lowered to 2, the marker bands of Fe(III) H23MP10 undergo shifts to positions (ν_4 = 1366 cm^{-1}, ν_3 = 1482 cm^{-1}, ν_2 = 1567 cm^{-1}, ν_{10} = 1615 cm^{-1}) corresponding to a pure high-spin ferric center. Indeed, the spectrum closely resembles that of the His-aquo form of MP8 at pH 2 (ν_4 = 1367 cm^{-1}, ν_3 =1484 cm^{-1}, ν_2 = 1567 cm^{-1}, ν_{10} = 1617 cm^{-1}).[16,17]

Addition of excess dithionite to argon-purged samples of Fe(III) H23 and Y23MP10 in pH 7 phosphate results in bright pink solutions of the corresponding Fe(II) forms. Resonance Raman spectra of Fe(II) H23 and Y23MP10 exhibit shifts in porphyrin marker frequencies consistent with the formation of low-spin ferrous heme centers.[14,15,27] The intense ν_4 oxidation state marker band shifts approximately 20 cm^{-1} to lower frequency upon reduction of both Y23 and H23MP10, appearing at 1351 cm^{-1}, with no detectable signal from unreacted Fe(III) species at 1368 cm^{-1}. The position of the spin-sensitive marker ν_2 at 1586 cm^{-1} in H23 and Y23MP10 suggests a six-coordinate Fe(II) in both cases.[14,15] The spectrum of Fe(II) Y23MP10 exhibits several moderately intense features in the low-frequency region that do not have counterparts in the spectrum of Fe(II) H23MP10; these features could indicate that there is a somewhat greater distortion of the porphyrin in Y23MP10.[29]

The ability of trypsin to accept a wide variety of incoming nucleophiles should facilitate the synthesis of a wide variety of novel heme peptides. In the X23MP10 series (X = H, Y, M), the ligand-binding behavior provides a benchmark for evaluation of the effects of folding on axial-ligand interactions in heme proteins; the histidine imidazole binds both Fe(III) and Fe(II) over a wide range of pH, tyrosine binding is seen only at high pH, and the thioether of methionine has very little affinity for Fe(III). In accord with observation,[30] our results suggest that histidine ligation will be favored in misfolded forms of cyt *c*. Although a tyrosine hydroxyl may be able to coordinate at high pH, it is unlikely that a methionine thioether will bind to Fe(III) in any unfolded state of the protein.

Acknowledgment. We thank Adrian Ponce for technical assistance and John Dawson for helpful discussions. D.W.L. acknowledges a fellowship from the Parsons Foundation. This work was supported by the NSF.

Supporting Information Available: Figures showing details of purification protocols, FPLC data, low-frequency regions of Raman spectra, and mass spectra (11 pages). See any current masthead page for ordering and Internet access instructions.

JA970103Q

(26) The X-band EPR spectrum of a frozen solution of H23MP10 (pH 2) shows a prominent g = 6 resonance characteristic of high-spin Fe(III) (Palmer, G. In *Iron Porphyrins, Part Two*; Lever, A. B. P., Gray, H. B., Eds.; Addison-Wesley Publishing Company: Reading, MA, 1983; pp 64−70). The electronic absorption spectrum of the low-pH form is identical with that of high-spin His-aquo MP8 (λ_{max} = 396 nm; Aron, J.; Baldwin, D. A.; Marques, H. M.; Pratt, J. M.; Adams, P. A. *J. Inorg. Biochem.* **1986,** *27*, 227−243).

(27) Spiro, T. G. *Adv. Protein Chem.* **1985**, *17*, 111−159.
(28) Wang, Y.; Van Wart, H. E. *Methods Enzymol.* **1993**, *226*, 319−373.
(29) These spectra are given in the Supporting Information.
(30) Babul, J.; Stellwagen, E. *Biopolymers* **1971**, *10*, 2359−2361.