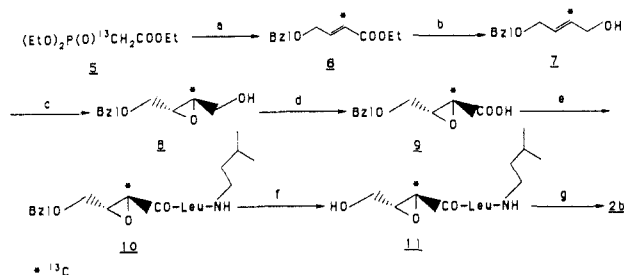


Scheme II. Synthesis of [<sup>13</sup>C-2]-Ep-475 (**2b**)

(a) BzIOCH<sub>2</sub>CHO, NaH, 1,2-dimethoxyethane, 25 °C, 30 min; 80% yield. (b) Diisobutylaluminum hydride, C<sub>6</sub>H<sub>6</sub>, 25 °C, 4 h, 84%. (c) Titanium tetraisopropoxide, (-)-diethyl tartrate, *tert*-butyl hydroperoxide,<sup>10</sup> CH<sub>2</sub>Cl<sub>2</sub>, -23 °C, 20 h, 88%. (d) O<sub>2</sub>/Pt black, AcOEt/aq. NaHCO<sub>3</sub> (1:1),<sup>11</sup> 25 °C, 5 days 82%. (e) L-Leu-NH[CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>]<sub>2</sub>,<sup>2b</sup> 1,3-dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, 0 °C, 1 h; then 25 °C 7 h, 58%. (f) H<sub>2</sub>/Pearlman's catalyst, EtOH, 25 °C, 6.5 h, 82%. (g) O<sub>2</sub>/Pt black, AcOEt/aq. NaHCO<sub>3</sub> (1:1),<sup>11</sup> 25 °C, 4 days; then Dowex 50W (H<sup>+</sup> form), 77%.

and DTT, and the <sup>13</sup>C NMR spectrum of the complex in H<sub>2</sub>O was taken. The <sup>13</sup>C NMR spectrum of the complex displays a new broad signal (line width = 35 Hz) at 76.5 ppm (Figure 1c). When an excess of inhibitor **2b** was added to the reaction mixture and then the NMR spectrum retaken, only the resonance at 52.5 ppm for C-2 of the epoxide increased (Figure 1d) which established that the new resonance at 76.5 did not arise from artifacts. The chemical shift of the new carbon resonance at 76.5 ppm (Figure 1c) shows that the new signal which is formed from the reaction of **2b** with papain corresponds to a secondary alcohol at C-2. Moreover, no peak near 50 ppm for the alternative alcohol product which would be formed from attack of C-2 in **2b** could be detected. A higher resolution difference spectrum, obtained from samples prepared by activating papain separately in the presence of labeled (**2b**) and unlabeled (**2a**) Ep-475 (Figure 1e), clearly showed the presence only of the 76.5-ppm resonance (Figure 1f). This latter experiment gave a better difference spectrum because activated papain at the high concentrations needed for the NMR spectra undergoes autolysis in the absence of inhibitors. These data establish that the active site thiol group in papain cleanly attacks the C-3 carbon of the epoxysuccinyl group in Ep-475 (**12**).

Our approach complements <sup>13</sup>C NMR techniques that have been used to characterize other enzyme-inhibitor interactions<sup>13-20</sup> and enzyme catalytic mechanisms,<sup>21,22</sup> and has led to the discovery that the product formed results from attack of the Cys-25 thiol group in papain on the C-3 carbon of the epoxide **2b** (Scheme I, a). This highly regioselective attack, coupled with the rapid inactivation only of cysteine proteinases by epoxysuccinic acid derivatives (apparent second-order rate constants approach 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup>),<sup>4</sup> suggests that the reaction of the Cys-25 sulfhydryl group with the epoxide moiety in E-64 and related derivatives is an enzyme-catalyzed process that occurs in the active site of cysteine proteinases. Now that the attachment site is known, molecular modeling studies of E-64 derivatives bound in the active site of

cysteine proteinases can be initiated in order to determine both the enzyme subsites that E-64 derivatives bind to prior to the alkylation reaction and the relationship between the catalytic and inhibitor mechanisms.

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### Formation of Nitridoiron(V) Porphyrins Detected by Resonance Raman Spectroscopy

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Formation of ferrylporphyrins is regarded as the most crucial step in the reaction cycle of cytochrome P-450.<sup>1</sup> A recent finding that liver microsomal cytochrome P-450-LM3,4 catalyzes functionalized nitrogen atom transfer<sup>2</sup> intra- as well as intermolecularly suggests that a multiple FeN bond similar to the ferryl FeO bond is involved in the reaction cycle. Nitridoiron porphyrins (formally written as N≡Fe(por)) may be regarded as models of such intermediate species. It is highly important therefore to prepare such compounds and characterize them by spectroscopic methods and to determine whether such nitridoiron porphyrins are formulated as Fe(V) porphyrins or Fe(IV) porphyrin π-cation radicals. Furthermore, it should be noted that only a few examples of iron(V) compounds are known thus far.<sup>3</sup>

Stable nitridomanganese(V) porphyrins<sup>4,5</sup> and nitridochromium(V) porphyrins<sup>6</sup> have been prepared by chemical oxidation as well as by photolysis<sup>7,8</sup> and their ν(M≡N) (ν is the stretching frequency) could be identified spectroscopically. Recently, Tsubaki et al.<sup>8</sup> observed ν(Mn≡N) of nitridomanganese(V) substituted myoglobin and horseradish peroxidase at 1010 and 1003 cm<sup>-1</sup>, respectively, by resonance Raman (RR) spectroscopy. However, no reports are available on nitridoiron porphyrins. In this communication we report the formation of N≡Fe(TPP) (TPP = tetraphenylporphine) by laser photolysis of the azido complex and its RR characterization for the first time.

Azido(tetraphenylporphinato)iron, N<sub>3</sub>Fe(TPP), and its <sup>54</sup>Fe, <sup>15</sup>N<sub>3</sub>, and N<sub>2</sub><sup>15</sup>N analogues were prepared by the method of Adams et al.<sup>9</sup> TPP, <sup>54</sup>Fe, and <sup>15</sup>N containing azides were purchased from Midcentury, Oak Ridge Laboratory, and Stohler Chemicals, respectively. Methylene chloride solutions of these azido complexes were evaporated on the surface of a cold tip and the films thus obtained were cooled to ≈30 K by a CTI Model 20/70 cryocooler. RR spectra were measured on a Spex Model 1403 double monochromator equipped with a Spex DM1B computer. The 514.5-nm line from a Spectra-Physics Model 2025 Ar<sup>+</sup> laser was used for photolysis and for RR excitation. Laser power levels up to 70 mW were applied to the samples through a cylindrical lens.

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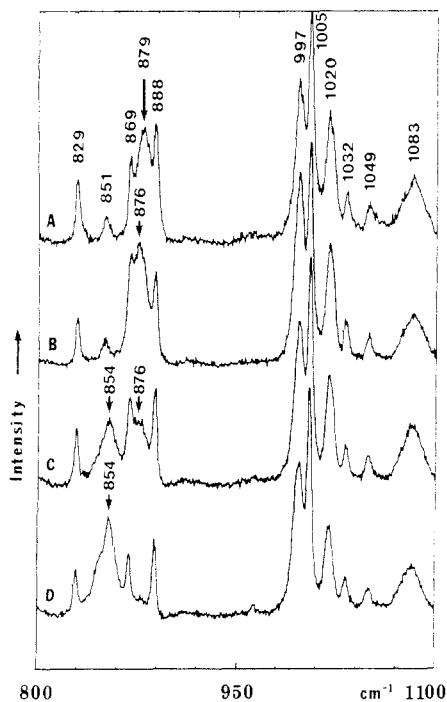


Figure 1. Resonance Raman spectra of photolysis products of (A)  $N_3^{54}Fe(TPP)$ ; (B)  $N_3Fe(TPP)$ ; (C)  $N_2^{15}NFe(TPP) + ^{15}NN_2Fe(TPP)$ ; and (D)  $^{15}N_3Fe(TPP)$ , thin films at  $\approx 30$  K, 514.5-nm excitation, 70 mW.

Parts A and B of Figure 1 compare the RR spectra of the photolysis products of  $N_3^{54}Fe(TPP)$  and  $N_3Fe(TPP)$  (Fe in natural abundance contains 91.7% of  $^{56}Fe$ ), respectively. The bands at 879 and 876  $cm^{-1}$  are not present in the RR spectra of the corresponding azido complexes. The upward shift of 3  $cm^{-1}$  by  $^{56}Fe/^{54}Fe$  substitution suggests that the 876- $cm^{-1}$  vibration involves the displacement of the Fe atom. Parts C and D of Figure 1 show the RR spectra of the photolysis products obtained from the  $N_2^{15}N$  and  $^{15}N_3$  azido analogues. It is seen that the 876- $cm^{-1}$  band is shifted to 854  $cm^{-1}$  by  $^{14}N/^{15}N$  substitution (Figure 1D) and that the photolysis product of the  $N_2^{15}N$  azido complexes exhibits two bands at 876 and 854  $cm^{-1}$  (Figure 1C). These observations provide definitive evidence that the above vibrations are due to the  $\nu(Fe\equiv N)$  of  $N\equiv Fe(TPP)$ . Simple diatomic calculations predict the isotopic shifts of +3 and -23  $cm^{-1}$  for  $^{56}Fe/^{54}Fe$  and  $^{14}N/^{15}N$  substitutions, respectively, for  $\nu(Fe\equiv N)$ . This is in perfect agreement with the observed values. The band at 876  $cm^{-1}$  cannot be attributed to a  $\mu$ -N dimer for several reasons. (1) It disappears above 150 K while the  $\mu$ -N dimers are stable at room temperature.<sup>10-12</sup> (2) With excitation at 514.5 nm no band was observed in the symmetric Fe-N-Fe stretching region (424 and 465  $cm^{-1}$  for  $(FeTPP)_2N$  and  $(FeTPP)_2N^+$ , respectively<sup>11,12</sup>). (3) A similar  $^{15}N$ -sensitive band could be observed with TMP (TMP = tetramesitylporphine)<sup>13</sup> at 873  $cm^{-1}$ . In this case steric hindrance prevents  $\mu$ -N bridge formation. (4) The antisymmetric Fe-N-Fe stretching frequency has been reported at 910 and around 1000  $cm^{-1}$  for  $(FeTPP)_2N$  and  $(FeTPP)_2N^+$ , respectively, in IR spectra.<sup>10,12</sup>

The abrupt drop in the  $\nu(M\equiv N)$  (Figure 2c) in going from the Cr(V) (1017  $cm^{-1}$ ),<sup>7</sup> Mn(V) (1049-52  $cm^{-1}$ )<sup>4,6,14</sup> to the Fe(V) (876  $cm^{-1}$ ) nitridoporphyrin may be accounted for in terms of the MO schemes shown in Figure 2a. The electron configuration shown there was proposed by Czernuszewicz et al.<sup>15</sup> to explain

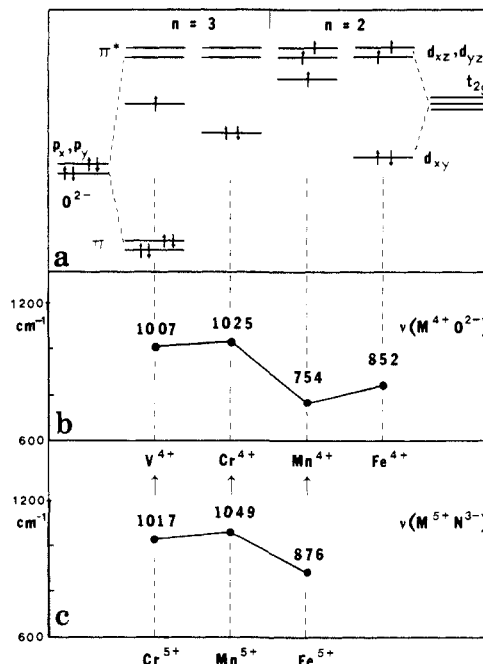


Figure 2. (a) Electron configuration of  $M^{4+}O^{2-}$  porphyrins (ref 15). (b) Variation of  $\nu(M^{4+}O^{2-})$  in  $M^{4+}O^{2-}$  porphyrins,  $M = V^{4+}$  (1007  $cm^{-1}$ )<sup>16</sup>,  $Cr^{4+}$  (1025  $cm^{-1}$ )<sup>17</sup>,  $Mn^{4+}$  (754  $cm^{-1}$ )<sup>15</sup> and  $Fe^{4+}$  (852  $cm^{-1}$ )<sup>18,19</sup>. (c) Variation of  $\nu(M^{5+}N^{3-})$  in  $M^{5+}N^{3-}$  porphyrins,  $M = Cr^{5+}$  (1017  $cm^{-1}$ )<sup>7</sup>,  $Mn^{5+}$  (1049-52  $cm^{-1}$ )<sup>4,6,14</sup> and  $Fe^{5+}$  (876  $cm^{-1}$ , this work).

the decrease in  $\nu(M=O)$  (Figure 2b) in going from the  $O=V(TPP)$  (1007  $cm^{-1}$ )<sup>16</sup>  $O=Cr(TPP)$  (1025  $cm^{-1}$ )<sup>17</sup> to  $O=Mn(TPP)$  (754  $cm^{-1}$ )<sup>15</sup> and  $O=Fe(TPP)$  (852  $cm^{-1}$ )<sup>18,19</sup>. According to their scheme the bond order is low ( $n = 2$ ) in the latter two compounds since electrons enter the antibonding  $d_{xz}$ ,  $d_{yz}$  orbitals. This is also supported by recent MO calculations for ferrylporphyrins.<sup>20</sup> Since the  $M^{VN^{3-}}$  system is one-electron deficient relative to the  $M^{IV}O^{2-}$  system, the  $Fe^{VN^{3-}}$  bond is iso-electronic with the  $Mn^{IV}O^{2-}$  bond. Thus, the electronic configuration of the  $Fe^{VN^{3-}}$  system may be  $(d_{xy})^1(d_{xz})^1(d_{yz})^1$  (high spin) or  $(d_{xy})^2(d_{xz})^1$  (low spin). In either case, the presence of electrons in the antibonding d-orbitals is expected to cause lowering of  $\nu(M\equiv N)$  in the Fe(V) complex. In the high-frequency region,  $N\equiv Fe(TPP)$  exhibits the oxidation/spin state sensitive band (band A)<sup>21,22</sup> at 1373  $cm^{-1}$  and the core size/spin state sensitive band (band D)<sup>21,22</sup> at 1576  $cm^{-1}$ . These frequencies are close to that of  $O=Fe(TPP)$ <sup>18</sup> (Fe(IV), low spin). This result rules out the possibility of a  $\pi$ -cation radical and favors the low-spin configuration mentioned above. More studies, both experimental and theoretical, must be made, however, before the definitive electronic configuration of nitridoiron porphyrin can be established.

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