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Structural Characterization of Nitrosylhemochromogen of Cooked Cured Meat: Implications in the Meat-Curing Reaction

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Cured meat pigment from cooked corned beef was isolated and completely characterized by IR and visible spectroscopies and shown to be identical with synthetic material that was further identified by fast atom bombardment mass spectrometry as the mononitrosyl species nitrosyliron(I) protoporphyrin. The ESR spectrum of this paramagnetic complex is reported for the first time. It was shown that this pigment can be formed from chloroiron(III) protoporphyrin by autoreduction with imidazole and nitric oxide. The same compound is formed by reacting nitric oxide gas with metmyoglobin followed by protein denaturation. These results lead to the proposal of a new mechanism for the meat-curing process involving (1) oxidation of myoglobin to metmyoglobin by nitrite, which is reduced to nitric oxide, (2) formation of the unobserved intermediate nitrosylmetmyoglobin, (3) rapid autoreduction to a nitrosylmyoglobin radical cation, (4) further reduction to nitrosylmyoglobin, and (5) formation of nitrosylhemochromogen (nitrosyliron(II) protoporphyrin) and incorporation of a second mole of nitrite into the denatured protein on heating.

The structure of the nitrosylhemochromogen pigment of cooked cured meat has long been a subject of dispute. This structure has been suggested as being either a five-coordinate mononitrosyliron(II) protoporphyrin complex or a six-coordinate dinitrosyliron(II) protoporphyrin complex.

Hornsey (1956) demonstrated that the characteristic red pigment of cooked cured meat could be extracted completely by an 80% acetone-water mixture. Electronic adsorbance and reflectance data of the pigments of cooked cured ham, heat-denatured nitric oxide hemoglobin, and the acetone extracts of these compounds were identical with each other and indicative of a low-spin ferrous coordination complex (Tarladgis, 1962). Attempts by Tarladgis (1962) to obtain an electron spin resonance spectrum of this pigment were unsuccessful under the conditions used. The absence of an ESR signal was assumed to indicate a diamagnetic six-coordinate dinitrosoyl heme complex 1 as the pigment of cooked cured meats. It was proposed that this pigment was formed on heating the uncooked cured meat pigment, nitric oxide myoglobin, resulting in denaturation of the protein and displacement of the globin by nitric oxide, which occupied both axial coordination positions of the iron.
Results of an experiment using 15N-labeled sodium nitrite to nitrosate myoglobin followed by Kjeldahl analysis indicate that twice as much labeled nitrogen was incorporated into myoglobin on heating, thus denaturing the protein, than into the unheated nitrosated system (Lee and Cassens, 1976). This was interpreted as evidence for the formation of a dinitrosylheme complex. The possibility of the second molecule of nitrite being incorporated into the protein rather than the heme was not taken into consideration however.

Maxwell and Caughey (1976) reported the preparation of a five-coordinate nitrosylimmon(II) protoporphyrin dimethyl ester. This complex was prepared by heating the bis(pyridine) iron(II) complex under vacuum followed by the introduction of nitric oxide gas. It was observed that 2 mol of pyridine was recovered and 1 mol of nitric oxide gas taken up for each molecule of pigment formed. The infrared spectrum of this complex exhibited a single solvent-dependent nitrosyl stretch in the 1600–1700-cm\(^{-1}\) region corresponding to a NO\(^-\) ligand state. The \(\nu_{NO}\) in noncoordinating solvents (CICH\(_2\)CH\(_2\)Cl, CHCl\(_3\), CCl\(_4\)) appeared in the 1669–1684-cm\(^{-1}\) region whereas the \(\nu_{NO}\) in 1-methylimidazole appeared at 1618 cm\(^{-1}\). For nitric oxide myoglobin, a single nitrosyl peak was observed at 1615 cm\(^{-1}\). The ESR spectrum of the five-coordinate nitrosyl heme complex 2 had a well-resolved triplet due to hyperfine splitting by the NO nitrogen. Spectra obtained in solvents providing a nitrogenous ligand as well as the spectrum of nitric oxide hemoglobin showed superhyperfine splitting of poorly resolved NO nitrogen hyperfine lines due to the nitrogenous ligand being in the sixth coordination position.

Bonnett et al. (1980) also obtained ESR spectra of actual cured meat samples as well as acetone extracts of these. The ESR spectrum of uncooked bacon showed both the poorly resolved six-coordinate feature of nitric oxide myoglobin as well as the well-resolved triplet of five-coordinate nitrosylhemes. On heating the sample, the six-coordinate feature disappeared and the triplet increased in intensity and sharpness. The acetone extract of cooked cured meat samples also had the well-resolved triplet expected for five-coordinate nitrosylhemes. Bonnett et al. (1980) also reported an ESR signal at \(g_c = 2.03\), which is unexplained and not extracted from the tissue by acetone.

The diamagnetic model compound dinitrosylmonon(II) tetraphenylporphyrin has also been reported (Wayland and Olson, 1974). The infrared spectrum of this compound exhibits nitrosyl stretching bands at 1780 and 1700 cm\(^{-1}\) corresponding to NO\(^+\) and NO\(^-\) ligand states, respectively. Evacuation of excess NO gas results in decomposition to the mononitrosyl complex and disappearance of the NO\(^+\) band.

Results of previous studies indicate that oxidation of myoglobin to metmyoglobin is the initial reaction occurring on addition of nitrite to meat, and the next reaction step involves bonding of nitric oxide to metmyoglobin to form an intermediate postulated to be nitrosylmetmyoglobin (Walters et al., 1967). The electronic spectrum of this complex is however more consistent with an iron(II) porphyrin species. LaMar and Del Gaudio (1977) have shown that iron(III) porphyrin complexes are readily autoreduced to the iron(II) species by coordinated ligands such as cyanide, \(\alpha\)-hexanethiol, and piperidine in the following manner:

\[
\text{PFer}^{\text{III}}L + \text{NO} \rightarrow \text{PFer}^{\text{II}}L + \text{NO}^+ + \text{NO}^-.
\]

This paper is a description of research to determine the structure of cooked cured meat pigment from corned beef which is compared with synthetic pigment. A new overall mechanism for the curing reaction is proposed. Data relative to results reported herein have been reported (Killday et al., 1985, 1987) and published in a thesis (Killday, 1986).

**MATERIALS AND METHODS**

**Instruments and Techniques.** The \(^1\)H FT NMR measurements were taken on a 90-MHz JEOL Model FX 90-Q equipped with a NM-PVT variable-temperature unit. Proton resonances are expressed (ppm) downfield from internal tetramethylsilane (TMS) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The Fourier transform infrared spectra (FT-IR) were recorded on a Nicolet 20 DXB and the UV-visible spectra on Perkin-Elmer ST spectrophotometer 576. FABMS data were obtained on a VG-70EQ mass spectrometer at 4 kV. For thin-layer chromatography, silica gel G (Merck) or HPTLC plates were used. Nitric oxide was obtained from Matheson Gas Products Inc. and chloroiron(III) protoporphyrin (chlorohemin, 97+%) and imidazole were purchased from Eastman Kodak Co. Myoglobin (2X crystallized equine heart) was obtained from the Calbiochem Co.

**Synthesis of Nitrosylmonon(II) Porphyrin via Dithionite Reduction.** A solution containing Na\(_2\)S\(_2\)O\(_4\) (6.6 g) in water (100 mL) was deoxygenated for 10 min with a stream of argon. A centrifuge tube containing chlorohemin (15 mg) in dimethyl sulfoxide (1.5 mL) was simultaneously deoxygenated with argon. The dithionite solution (0.2 mL) was added to the chlorohemin solution, immediately forming a red color. Nitric oxide gas in a stream of argon was then bubbled through this for 1 min, intensifying the red color. Then, 12 mL deoxygenated water was added to precipitate the red nitrosylmonon(II) protoporphyrin. The product was washed at the centrifuge with three more 12-mL portions of water and dried under vacuum overnight. A portion of this was dissolved in acetone. TLC on silica gel developed with ethyl acetate–methanol (1:1) showed a single red compound at \(R_f\) 0.55. Visible spectrum (acetone): \(\lambda_{\text{max}}\) 481, 543, 564 nm. FABMS (major fragments): 615 (100%) [M – HNO]\(^+\), 647
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Extraction and Isolation of the Cooked Cured Meat Pigment from Corned Beef. Precoked corned beef was purchased from a local delicatessen, and extractions were performed in a darkened room illuminated by a weak red light. All solvents were deoxygenated by bubbling with a stream of argon gas. Typical extractions consisted of mincing 150 g of corned beef with 200 mL of acetone and filtering through a pad of Celite filter aid, resulting in a bright red solution (Hornsey, 1956). The acetone was then removed on a rotary evaporator, leaving approximately 30 mL of residual water containing a suspension of fat and pigment. This was extracted with two 30-mL portions of ethyl acetate, again forming a bright red solution. The ethyl acetate was removed on a rotary evaporator, leaving a red oil containing red solid particles. Thin-layer chromatography on silica gel developed with ethyl acetate-methanol (1:1) revealed a small amount of brown pigment at Rf 0.20 and a red pigment at Rf 0.55, and staining with phosphomolybdic acid showed a large amount of a blue-staining compound at Rf 0.65. When an ethyl acetate solution of this mixture was exposed to light and air, the solution turned green. The red pigment decomposed to form the more polar brown pigment analyzed by TLC. 

Formation of Nitrosyliron(II) Protoporphyrin by Autoreduction with Imidazole and Nitric Oxide. Chloroiron(III) protoporphyrin dimethyl ester (3 mg) and imidazole (10 mg) were dissolved in DMSO (2 mL). This solution was deoxygenated with argon gas and heated to 100 °C in a water bath. Nitric oxide gas was then bubbled through the solution for 30 s, followed by argon gas (3 min). The reaction mixture was cooled, and chloroform (3 mL) was added. The chloroform layer was extracted with three 10-mL portions of water to remove the imidazole and other imidazolium products. A few drops of the bright red chloroform solution was deposited on a NaCl plate, the solvent evaporated by a stream of N₂ and the FT IR spectrum obtained. IR (thin film): 1735 (ester C=O), 1662 (N=O), 1355, 1257, 1196, 1165, 1082, 1057, 954, 834, 796, 704 cm⁻¹. This compound was unstable in solution and quickly decomposed to a brownish green pigment.

Reaction of Metmyoglobin with Nitric Oxide Gas: Visible Spectroscopy. A stock solution of 0.5% (0.3 mM) metmyoglobin in 0.1 M phosphate buffer (pH 6.5) was prepared. Three solutions were prepared from this containing (a) stock solution only, (b) stock solution + 15 mM sodium ascorbate, and (c) stock solution + 15 mM Na₂S₂O₅. The solutions were deoxygenated by sonication under vacuum and placed under 1 atm of nitric oxide gas. Aliquots of each solution were removed and their visible spectra obtained from 500 to 650 nm: (a) λₚₐₓ 533, 576 nm; (b) λₚₐₓ 550, 583 nm; (c) λₚₐₓ 551, 582 nm. When solution (a) was allowed to stand for 16 h at room temperature in the dark, the spectrum changed to λₚₐₓ 550 and 580 nm. 

Isolation of Nitrosyliron(II) Protoporphyrin from a Metmyoglobin–Nitric Oxide Solution. Three milliliters of a saturated metmyoglobin solution in 0.1 M phosphate buffer (pH 6.5) was deoxygenated by sonication under vacuum, placed under an atmosphere of nitric oxide gas, and shaken for 3 min. Deoxygenated acetone (15 mL) was added and the solution shaken. A white precipitate appeared (denatured protein) and the red heme pigment extracted into the acetone-water solution. The denatured protein was removed by centrifugation and the acetone removed on a rotary evaporator until red pigment precipitated from solution. Deoxygenated water (10 mL) was added and the pigment collected by centrifugation and dried under vacuum. The pigment was dissolved in 2 drops of deoxygenated dimethyl sulfoxide, the solution deposited on a NaCl plate, and the DMSO removed under vacuum. TLC on silica gel developed with ethyl acetate–methanol (1:1) showed a single red compound at Rf 0.55.

Preparation of Chloroiron(III) Protoporphyrin Dimethyl Ester from the Diacid. The method used by Wang et al. (1958) for making the chlorohemin diethyl ester was followed except for the substitution of methanol for ethanol.

Preparation of Nitrosyliron(II) Protoporphyrin Dimethyl Ester. Chloroiron(III) protoporphyrin dimethyl ester (6 mg) was dissolved in chloroform (2 mL). This solution was deoxygenated with argon gas, and a solution containing Na₂S₂O₅ (100 mg) and NaN₃O₅ (100 mg) in deoxygenated water (3 mL) was added and the suspension shaken vigorously for 2 min. The layers were separated under gravity, and the chloroform layer, initially dark brown, turned bright red due to formation of the nitrosyliron(II) protoporphyrin dimethyl ester. Visible spectrum (CHCl₃): λₚₐₓ 485, 546, 568 nm. IR (thin film): 1736 (ester C=O), 1665 (N=O), 1436, 1250, 1197, 987, 914, 840, 730 cm⁻¹. This compound was unstable in solution if exposed to air and quickly decomposed to a brownish green pigment. It was however stable for several months in the chloroform solution if kept covered with the aqueous dichloromethane–nitrile solution and tightly capped.

RESULTS AND DISCUSSION

Synthesis, Isolation, and Characterization of Nitrosylhemochromogen. Attempts to synthesize nitrosylhemochromogen by the reductive nitrosylation method
of Bonnett et al. (1980) were only marginally successful. Low yields were obtained, and the method was time-consuming and used an excessive amount of nitric oxide gas. An alternate synthesis involving dithionite reduction in dimethyl sulfoxide–water was devised. This synthesis was much more efficient and produced pure product in >90% yields. The infrared spectrum of this compound had a nitrosyl stretch at 1656 cm\(^{-1}\). This is consistent with a bent NO\(^-\) ligand state. No absorption bands corresponding to a second coordinated nitrosyl ligand were present in the 1900-cm\(^{-1}\) region. The intensity of \(\nu_{\text{NO}}\) is a definitive marker for extent of formation and decomposition of the product.

To conclusively prove the structure of this compound, it was subjected to fast atom bombardment mass spectrometry (FABMS). This soft ionization technique can produce molecular ion peaks from extremely labile compounds. The FABMS results are shown in Figure 1. A mononitrosylheme complex would have a molecular ion of 646 amu. The cluster peaks seen correspond with a mononitrosyl complex as opposed to a dinitrosyl complex, which would show clusters 30 amu greater. The infrared and mass spectral data confirm the structure of this synthetic complex as being nitrosyliron(II) protoporphyrin (2).

Isolation of this pigment was quite difficult due to its instability in the presence of air and light. Although other investigators have stabilized the pigment in crude extracts by adding reductants such as ascorbate or dithionite, this method was avoided due to the possible alteration of the pigment. The blackened product obtained on washing with toluene was readily soluble in DMSO, forming a bright red solution. The black color was probably due to a coating of oxidized decomposition product being formed on the surface of the microcrystals. Complete isolation of this pigment from cured meat has not previously been reported nor has the infrared spectrum. When the spectrum was compared with the spectrum of further oxidized pigment, it was seen that absorption bands at 1350, 1287, 1267, 1203, 936, and 818 cm\(^{-1}\) were due to traces of decomposition product. Comparing the IR spectrum with that of the synthetic pigment as well as visible spectra and \(R_f\) values obtained from thin-layer chromatography, we see that the two compounds are identical. This provides strong evidence that the pigment of cooked cured meat is nitrosyliron(II) protoporphyrin (2).

The \(^1\)H NMR spectrum of nitrosyliron(II) protoporphyrin has not previously been reported. Extreme difficulty was met in obtaining a meaningful spectrum of this compound. Some of the problems faced were instability, insolubility, aggregation, and strong temperature and concentration dependence. Judicious choice of parameters such as shortened pulse delay and acquisition time, which enhance the paramagnetic resonances, was required.

The \(^1\)H NMR spectrum of synthetic nitrosyliron(II) protoporphyrin at 34, 50, and 70 °C is shown in Figure 2. Although other temperatures were studied, 50 °C gave the best resolution. In making assignments, it is probably safe to assign the resonances seen in the \(\delta 4.8-6.1\) range to the 1,3,5,8 methyl resonances and the \(\delta,\gamma \text{-CH}_2\)'s. The resonance at \(\delta 1.18\) is most likely the \(\delta,\gamma \text{-CH}_2\)'s due to its sharpness and intensity. The upfield resonances cannot however be assigned with any certainty with the information available. In order to unambiguously assign all resonances, selective deuteration would be necessary.

The nitrosyliron(II) protoporphyrin is unique among the iron porphyrins previously studied in that it is isoelectronic with low-spin cobalt(II) compounds, with the \(T_{2g}\) orbitals fully occupied and the unpaired electron in a primarily \(d_{x^2-y^2}\) orbital. The evidence for this is that significant downfield shifts are not observed as is the case for complexes containing unpaired spin density in the \(x\)-bonding \(d_{yz}\) and \(d_{xz}\) and the \(\pi\)-bonding \(d_{x^2-y^2}\) orbitals.

**Autoreduction of Chloroiron(III) Protoporphyrin:**

**\(^1\)H NMR Studies.** Addition of excess imidazole to chlo-
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nances characteristic of diamagnetic low-spin iron(II) protoporphyrin complexes were also not seen. It was therefore concluded that the nitrosyliron(II) protoporphyrin complex was formed and that the paramagnetically broadened resonances of this compound were obscured by the imidazole, water, and solvent resonances. The thin-layer chromatogram showed this pigment to have an $R_f$ equivalent to that of synthetic and extracted nitrosyliron(II) protoporphyrin. A control was also analyzed in order to determine if nitric oxide would reduce chlorohemin in the absence of imidazole. The $^1$H NMR spectrum of hemin was obtained, followed by introduction of nitric oxide gas and heating to 100 °C. On cooling to 31.7 °C and rerunning the spectrum, no significant changes were found.

**Formation of Nitrosyliron(II) Protoporphyrin by Autoreduction of Metmyoglobin.** Introduction of nitric oxide gas into a metmyoglobin solution resulted in formation of a red complex exhibiting $\lambda_{\text{max}}$ 533 and 576 nm. After 12 h under an atmosphere of NO(g), the visible spectrum shifted to $\lambda_{\text{max}}$ 550 and 580 nm, equivalent to that of nitrosylmyoglobin prepared in the presence of ascorbate.

Addition of acetone to a saturated metmyoglobin/nitric oxide phosphate buffer solution resulted in denaturation and precipitation of the protein and extraction of pigment into the acetone solution. This pigment was identified by

**Figure 4.** $^1$H NMR spectrum of chloroiron(III) protoporphyrin (3 mg) + imidazole (10 mg) + nitric oxide (1 atm) in 1 mL of [D$_6$]DMSO after heating at 70 °C ($T = 31.5$ °C).

**Figure 5.** Proposed reaction mechanism for the meat-curing reaction.
TLC and FT IR as nitrosyliron(II) protoporphyrin. It is significant to note that this pigment was readily formed without the addition of exogenous reductant. Addition of acetone to a solution of metmyoglobin also resulted in denaturation of the protein. The pigment, however, remained with the denatured protein and did not extract into the acetone-water solution.

Discussion. From these results, a new theoretical sequence for the meat-curing reaction can be formulated that helps explain the inconsistencies of previously proposed mechanisms. This sequence is shown in Figure 5. The initial reaction involves oxidation of deoxymyoglobin, the predominant pigment in antemortem muscle tissue, to metmyoglobin. In this process, nitrite is reduced to nitric oxide that then reacts with metmyoglobin. The next step involves either (1) formation of nitrosylmetmyoglobin followed by rapid autoreduction to the nitrosylmyoglobin radical cation 3 or (2) a conjugated mechanism involving simultaneous NO coordination and autoreduction to 3. In either case, the iron(III) species nitrosylmyoglobin is not observed and the complex previously thought to be nitrosylmetmyoglobin is actually the iron(II) nitrosylmyoglobin radical-cation species 3. Evidence for this is as follows: (1) Imidazole readily autoreduces iron(III) protoporphyrin in the presence of nitric oxide. (2) Acetone extraction of this complex yields nitrosyliron(II) protoporphyrin. (3) The visible spectrum of this complex is consistent with a low-spin iron(II) protoporphyrin complex and is in fact nearly identical with that of nitrosylmyoglobin—only shifted 10 nm to shorter wavelength. This shift to higher energy would be expected as the ligand charge increased.

Since no exogenous reducing compounds were added to the model system, the slow reduction of 3 to nitrosylmyoglobin (4) is apparently due to reducing systems within the protein itself. An alternative explanation for this would be migration of the charge to an adjacent histidine residue. This would explain incorporation of a second mole of nitrite as discussed below.

When nitrosylmyoglobin is heated, the protein is denatured and detached from the heme and a second mole of nitrite is incorporated (Lee and Cassens, 1975). Since nitrosylhemochromogen has been demonstrated to be a mononitrosyl complex, the second nitrite must be bound to the denatured protein. The nature of this complex as well as its mechanism of formation is a matter of critical concern. One possibility is that the nitrosylmyoglobin contains a protein radical 4 that upon denaturation reacts with nitrite or an oxide of nitrogen. This hypothesis is supported by the precise stoichiometry of the reaction. Further work may be needed to obtain absolute proof of the proposed mechanism.

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LITERATURE CITED


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