C84

FURTHER EVIDENCE FOR A MONONITROSYLHAEM COMPLEX OF THE COOKED CURED-MEAT PIGMENT

F. SHAHIDI and R.B. PEGG
Department of Biochemistry, Memorial University of Newfoundland, St. John's, NF, A1B 3X9, Canada and
PA Pure Additions, Inc., c/o Memorial University of Newfoundland, St. John's, NF, A1B 3X9, Canada. Fax (709) 737-4000

INTRODUCTION

The structure of the nitrosylprotohaem pigment of cooked cured meat has long been a subject of controversy. Based on numerous spectroscopic investigations of nitrosylhaem complexes, it is believed that during thermal processing of nitrite-cured meat, the globin portion of nitrosylmyoglobin denatures and subsequently detaches itself from the haem moiety. The resultant pigment is either a five-coordinate mononitrosylprotohaem complex or a six-coordinate dinitrosylprotohaem compound, if it acquires a second molecule of nitric oxide.

Our interest in the chemical nature of the cooked cured-meat pigment (CCMP) stems from our investigations into nitrite-free meat curing systems. We have reported the preparation of CCMP from haemin (Shahidi *et al.*, 1994) in the presence of reductants and NO. This pigment, preformed outside of the meat matrix, is used as part of a composite package for nitrite-free curing of meat products, but its chemical nature has not been adequately elucidated. Bonnett *et al.* (1980) attempted to characterize the pigment of cooked cured meat, nitrosylprotohaem, from the reaction of NO with protohaem dimethyl ester and with methoxyiron (III) protoporphyrin dimethyl ester. The EPR spectrum of nitrosylhaem in an acetone glass showed a triplet signal due to hyperfine splitting by a single axial nitrogenous ligand of NO indicating a pentacoordinate nitrosylhaem system (*i.e.* g_1=2.102, g_2=2.064, g_3=2.010). Killday *et al.* (1988) isolated and characterized an extract of CCMP from thermally processed corned beef by IR and VIS spectroscopies and thin-layer chromatography. These authors identified the pigment by fast atom bombardment mass spectrometry as being a mononitrosyl ferrous protoporphyrin. Jankiewicz *et al.* (1994) through UV-VIS absorption studies provided further support to the view that the CCMP is a mononitrosyl derivative of haem. The purpose of this study was to investigate the chemical nature of the CCMP *via* a novel titration methodology which uses a spectrophotometric procedure to detect the endpoint.

MATERIAL & METHODS

A cylindrical glass cuvette was filled with 3000 µL of a 0.04 M Na_2CO_3 solution. To the cuvette, a 75 µL aliquot of a 3.5 mg/mL haem solution dissolved in the carbonate solution was added. A few crystals of sodium dithionite were added also to reduce the Fe(III) haem to its ferrous state. The cuvette was capped with a rubber septum and nitrogen was purged through the head space gases for *ca.* 30 s to remove any traces of oxygen. The absorption spectrum of the reduced haem solution was recorded at 450-650 nm using a Hewlett Packard 8452A diode array spectrophotometer (Hewlett Packard Canada, Ltd., Toronto, ON). Sodium carbonate solution was used for background subtraction. A gentle stream of NO was then bubbled into the system for *ca.* 10 s, thereby forming the nitrosyl haem analog, and its absorption spectrum was recorded.

A saturated solution of NO was prepared in a test tube of deoxygenated water. The tube was covered with Parafilm® in an effort to prevent penetration by oxygen, and NO was purged into the system for 20 min *via* a syringe needle. A small magnetic stirrer had been placed in the bottom of the tube to prevent supersaturation of NO in the medium. A fresh preparation of the reduced haem system was titrated with 25 or 50 µL aliquots of the NO solution. The absorption spectrum was monitored at 450-650 nm. The titration proceeded until it was believed that all reduced haem had been converted to nitrosylprotohaem or dinitrosylprotohaem. At this time, NO was bubbled into the cuvette and the absorbance spectrum then read to ensure that the reaction had gone to completion.

RESULTS & DISCUSSION

The maximum change in absorbance between the spectra of equal concentrations of reduced haem and its nitrosyl derivative occurred at 486 nm. Rather than bubbling NO directly into a fresh solution of reduced haemin to obtain the nitrosylhaem complex, aliquots of the saturated solution of NO were titrated into the air-tight cuvette *via* a Hamilton syringe, and the change in absorbance at the 486 nm band was monitored.

The basic equation for the reaction of haemin with NO in a protic solvent such as water or methanol, and in the presence of a reductant, is as follows:

$$ClFe^{III}P + 2\ NO \xrightarrow{H_2O} ONFe^{II}P + HONO + HCl,$$ where P = porphyrin dianion {adapted from Bonnett *et al.*, (1980)}

Data provided by Young (1981) showed that the concentration of a saturated solution of NO in water at standard pressure and a temperature of 293 K is 2.02 x 10^{-3} M in NO. Based on the number of moles of haemin present in the model system and the stoichiometry of the above reaction, it was determined that *ca.* 400 µL of the NO solution would be required to reach the endpoint of the titration if CCMP was a mononitrosyl haem complex; more would be needed if the pigment was dinitrosylprotohaem. Based on data presented in Table 1, 350-400 µL of NO solution were required to reach the endpoint of the titration, thereby suggesting that only one nitrosyl group is ligated to the reduced haem. These results are in agreement with those of Killday *et al.* (1988) and Jankiewicz *et al.* (1994) who suggested that a 1:1 complex between NO and reduced haem is formed.

Table 1. Change in Absorbance Upon Titration of Reduced Haem System with NO.

Volume of NO solution added (µL)	ΔAbsorbance (486 nm)[1] (corrected for dilution)	Volume of NO Solution Added (µL)	ΔAbsorbance (486 nm)[1] (corrected for dilution)
0	0.6209	300	0.9911
25	0.6820	350	0.9950
50	0.7909	400	1.001
75	0.8603	450	0.9974
100	0.9105	500	0.9962
125	0.9357	550	1.005
150	0.9597	600	0.9987
175	0.9725	650	1.002
200	0.9861	650 + NO gas	1.004
250	0.9896		

[1]ΔAbsorbance at 486 nm is between that of the nitrosylhaem complex formed and the reduced haem.

Further evidence for a mononitrosylhaem complex as the pigment of nitrite-cured meat comes from EPR studies by several authors. The EPR spectral parameters of an acetone extract of a sample of CCMP were compared to those of nitrosylprotohaem dimethyl ester investigated by Bonnett *et al.* (1980) and to $Fe^{II}TPP(NO)$ reported by Wayland and Olson (1974). In all cases examined, the EPR parameters of these systems were similar and possessed characteristics recognized as those of a pentacoordinate nitrosylhaem complex; that is, the EPR spectrum of CCMP in an acetone glass showed ^{14}N hyperfine splitting in the g_3 region with a_3 of 17.1G (Shahidi *et al.* 1994). Together with the EPR studies cited, the titration experiment reported above, provides strong evidence for a mononitrosyliron (II) complex for the cooked cured-meat pigment.

REFERENCES

Bonnett, R., Chandra, S., Charalambides, A.A., Sales, K.D. and Scourides, P.A. 1980. Nitrosation and nitrosylation of haemoproteins and related compounds. Part 4. Pentaco-ordinate nitrosylprotohaem as the pigment of cooked cured meat. Direct evidence from E.S.R. Spectroscopy. *J.C.S. Perkin I* 1706-1710.

Killday, K.B., Tempesta, M.S., Bailey, M.E. and Metral, C.J. 1988. Structural characterization of nitrosylhemochromogen of cooked cured meat: Implications in the meat-curing reaction. *J. Agric. Food Chem.* 36:909-914.

Jankiewicz, L., Kwaśny, M., Wasylik, K. and Graczyk, A. 1994. Structure studies on the nitrosyl derivative of heme. *J. Food Sci.* 59:57-59.

Shahidi, F., Pegg, R.B., Gogan, N.J. and DeSilva, S.I. 1994. The cooked cured-meat pigment - ESR studies. In: Proceedings of the 40th International Congress on Meat Science and Technology, The Hague, The Netherlands, August 28 - September 2.

Shahidi, F., Pegg, R.B. and Sen, N.P. 1994. Absence of volatile *N*-nitrosamines in cooked nitrite-free cured muscle foods. *Meat Sci.* 37:327-336.

Wayland, B.B. and Olson, L.W. 1974. Spectroscopic studies and bonding model for nitric oxide complexes of iron porphyrins. *J. Am. Chem. Soc.* 96:6037-6041.

Young, C.L. 1981. Nitric oxide. In *Solubility Data Series. Volume 8. Oxides of Nitrogen.* International Union of Pure and Applied Chemistry. Pergamon Press, Oxford, UK, pp. 260-351.