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A novel derivative of the prosthetic group heme d_1: *S*-methylporphyrindione d_1

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Several derivatives of heme d_1 have been characterized by ultraviolet-visible, NMR, and mass spectrometry. Most arise from side reactions during the isolation of d_1 from the enzyme. One, however, has now been shown to correspond to the replacement of a meso proton by an *S*-methyl group. Since the prophyrin is not exposed to *S*-methyl-containing reagents during its isolation, this raises hypotheses that it has its origin in vivo.

Introduction

Heme d_1 is the unusual prosthetic group of cytochrome cd_1, which functions as the dissimilatory nitrite reductase in many common denitrifying bacteria [1]. It is the iron chelate of a porphyrindione skeleton. Although there is now little doubt of the correct structure for d_1 following the total synthesis of the porphyrindione by Wu and Chang [2], there have remained several open questions concerning prophyrins of unknown structures that appeared during the isolation and characterization of natural d_1. Over a period of time it has been possible to accumulate these minor products and finally characterize them. The structures are most readily interpretable in terms of simple side reactions that have transformed authentic d_1 during its isolation and purification. But one compound, to be discussed here, offers some insight into a minor but unusual reaction that may be occurring in vivo.

Materials and Methods

Isolation and purification procedures for heme d_1 and the corresponding free base, methyl ester derivative and spectroscopic conditions for ultraviolet-visible, infra-red, and NMR spectroscopy have been described [3]. Minor fractions isolated during the purification of the major d_1 porphyrin have been accumulated and now characterized by NMR and high-resolution mass spectrometry. The latter technique is especially valuable when characterizing compounds available only in trace amounts. Mass spectral data were obtained on a VG Instruments AutoSpec. Heme and porphyrin d_1 are relatively polar tetrapyrroles and give poor electron impact spectra off a solids probe even at high temperatures. However, they give intense protonated molecular ions in LSIMS (liquid secondary ion mass spectrometry), also called FAB (fast atom bombardment). Some matrices for porphyrin analyses have been described [4]. In addition we have found that 3-nitrobenzyl alcohol containing 1% by volume trifluoroacetic acid is an excellent matrix for both polar and nonpolar porphyrins, and for the relatively polar d_1 derivatives, glycerol with 1% trifluoroacetic acid is also useful. Sample sizes of 0.5 to 1 μg afforded intense parent ions $(M+H)^+$ on standard LSIMS probes, and samples as small as 40 ng produced usable spectra in dynamic, flowing LSIMS techniques [5]. For exact mass determinations, poly(ethylene glycol) was used as internal reference. Electron microprobe analyses were performed by McCrone Associates, Westmont, IL.

Results and Discussion

During the isolation of d_1 a minor component has been repeatedly observed and termed the 'desmesoporphyrin' [6]. Because of its small amount and variable yields during different isolation steps, only an approximate upper limit can be given for its quantity. It is definitely less than 5%, and probably less than 1%, of the native d_1 in any isolated sample. Because of its low abundance it is only detectable in relatively

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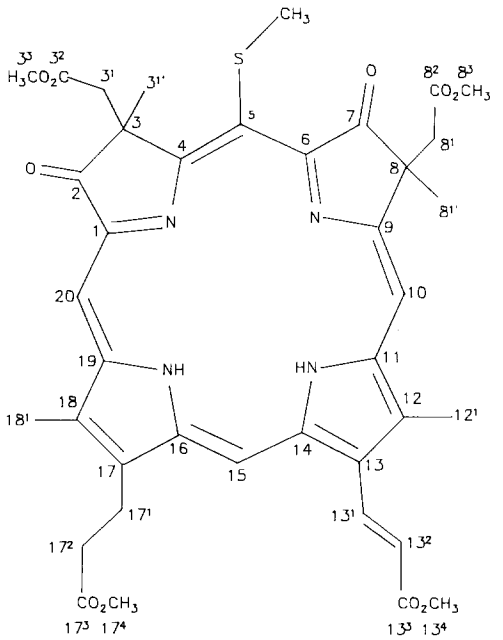
large-scale isolations (which for d_1 means isolations beginning with 5–10 μmol of starting enzyme) and copurifies with native d_1 until the final HPLC chromatography of the metal-free, esterified porphyrins. Its proton NMR spectrum has been reported [6] and is highly similar to native d_1 with three outstanding differences. One of the four meso proton singlets is absent (hence its designation); an AX spin subsystem appears with a large coupling constant of 17.6 Hz (suggestive of geminal coupling); and a new and extra three proton singlet appears at 2.54 ppm. High-resolution LSIMS mass spectrometry indicated an exact mass of 759.2674 $\pm$ 0.0024 (four trials) [6]. Several highly speculative structures were considered and one candidate proposed for this compound (see structure 4, Ref. 6). The candidates considered were based upon assuming that d_1 had undergone some in vitro side reaction during isolation. None gave a good match to the experimental mass spectrum. They required a substantial disruption of the core π-electron bonding in the porphyrindione, and this was not consistent with the observation that the ultraviolet-visible spectrum of the compound was actually highly similar to native d_1.

A major clue to this problem compound came from the structure of phenyl d_1. Cyt cd_1 is susceptible to suicide inactivation by substituted hydrazines acting as electron donors. Phenyl d_1 was isolated and characterized as the product formed when phenylhydrazine is used as substrate [7]. By an unknown radical-based mechanism, a meso proton is lost from native d_1 and replaced by a phenyl ring regionspecifically at the 5 position (IUB-IUPAC tetrapyrrole nomenclature). Due to the presence of the larger phenyl substituent, rotation of the aromatic ring and the neighboring acetate is hindered, and the acetate becomes an AX spin system with a geminal coupling constant of 18.4 Hz. In fact, the proton NMR spectrum of phenyl d_1 is highly similar to that of the 'demesoporphyrin', except that the former has phenyl protons and the latter has the unexplained singlet at 2.54 ppm.

Since the most puzzling data for the desmesoporphyrin was its exact mass, this was remeasured by high resolution (nominal 10,000) LSIMS in a variety of matrices. The result (759.269, standard deviation 0.002, 11 trials) was consistent with the previous data and still cannot be explained by any combination of adducts consisting of only C, H, and O. However, with a single sulfur atom, the empirical formula $C_{39}H_{42}N_4O_{10}S$ plus H^+ from the matrix (calc. 759.270) agrees within the capabilities of the technique. Treatment of the material with hydrogen peroxide in ethanol (final 3%) just prior to addition to the LSIMS matrix, caused a decrease or disappearance of the 759 $M+H^+$ ion, and the appearance of a strong ion of mass 775, corresponding to addition of oxygen to the compound by the generation of a sulfoxide. The same treatment applied



Scheme 1

to native d_1 did not lead to any new peaks and only the parent 713 mass peak was observed. The presence of sulfur was independently confirmed by electron microprobe. The technique is not definitive for establishing empirical formula, but it did confirm the presence of sulfur in the range of 3–7% by weight, which is wholly consistent with the mass spectral results. The scheme 1 fits all the data. The *S*-methyl group was assigned to the 5 meso position based upon analogy to the phenyl-d_1 derivative. This does not completely rule out the other meso positions. From the phenyl d_1 results, it is known that meso substitution does not radically perturb the visible spectrum and the electronegative *S*-methyl group converts an acetate AB subspectrum into AX with geminal coupling around 18 Hz.

It is difficult to invoke an in vitro side reaction during the isolation of d_1 as being responsible for 1, because at no point is the sample exposed to external sulfur reagents, in particular *S*-methyl groups. A possible explanation is that the enzyme Cyt cd_1 undergoes a rare but natural suicide inactivation event similar to the phenylhydrazine-promoted reaction. The *S*-methyl group could come from a variety of endogenous sources, including, for example, a methionine residue within the protein itself. Thus, compound 1 could be a landmark for a natural degradative pathway in vivo.

Other derivatives of d_1 that appear during its isolation have also now been characterized. Heme extracts prepared with acidic organic solvents invariably contain some small percentage, 5–20% of the iron-free porphyrin. Apparently the iron coordination is not as strong as with protoporphyrin IX systems. Experiments on heme extracts need to be considered in light of this

background of free base porphyrin. The porphyrin has an extraordinary affinity for copper and forms chelates with even trace amounts present in high purity solvents. For native d_1 and any of its derivatives, there has been a small but consistent background of Cu derivatives. These do not afford easily interpretable NMR spectra because of the paramagnetism of Cu(II), but can be identified in mass spectra as $M + 63$ and $M + 65$ ions. Esterification of d_1 in mineral acid-methanol leads to a slow addition of methanol across the acrylate double bond (observed mass, 745.311; calc. for $C_{39}H_{45}N_4O_{11}$, 745.308). A blue pigment was obtained during isolation of d_1 [3,6]. This has been too unstable to obtain any direct NMR or mass spectral data. However, mild air oxidation causes the compound to become green and convert to the normal visible, NMR, and mass spectra (after conversion, observed mass, 713.280; calc. for $M + H^+$ for d_1, 713.282) of native d_1. The blue pigment may be a reduced oxidation state of the porphyrin ring, perhaps the hydroquinone version of the porphyrin dione, generated in small amounts during the iron removal reaction which uses ferrous iron in excess.

These compounds account for all the porphyrins that we have observed during extensive isolation studies on the d_1 prosthetic group. The results afford some insight into the chemical reactivity of d_1, and lend confidence that nothing major has been overlooked.

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References

1 Payne, W.J. (1981) Denitrification, John Wiley & Sons, New York.
2 Wu, W. and Chang, C.K. (1987) J. Am. Chem. Soc. 109, 3149–3150.
3 Timkovich, R., Cork, M.S. and Taylor, P.V. (1984) J. Biol. Chem. 249, 1577–1585.
4 Musselman, B.D., Watson, J.T. and Chang, C.K. (1986) Org. Mass Spectrom. 21, 215–219.
5 Caprioli, R.M. and Moore, W.T. (1990) Methods Enzymol. 193, 214–237.
6 Chang, C.K., Timkovich, R. and Wu, W. (1986) Biochemistry 25, 8447–8453.
7 Yap-Bondoc, F. and Timkovich, R. (1990) J. Biol. Chem. 265, 4247–4253.