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# Cytochrome *c* oxidase models. Dinuclear iron/copper complexes derived from covalently modified deuteroporphyrins

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**Abstract**

The deuterohemin complex obtained by covalent modification of the propionic acid side chains of the porphyrin with a glycyl-L-histidine methyl ester residue and a bis(benzimidazole) residue, respectively, is reported. The bis(benzimidazole) arm can be used as a ligand for a copper(II) ion, giving an heterodinuclear complex that mimics the dioxygen reduction center found in cytochrome *c* oxidase. The relevance of the present model system is shown by the smooth reactivity that the reduced form of the heme/copper complex exhibits to dioxygen. The reaction, in fact, proceeds nondestructively even at room temperature to give the fully oxidized Fe(III)/Cu(II) species.

*Keywords:* Cytochrome *c* oxidase models; Dinuclear heme/copper complexes; Deuteroporphyrin modification; Dioxygen activation

## 1. Introduction

Cytochrome *c* oxidase is the terminal enzyme in the electron transfer chain that functions as catalyst for the four-electron reduction of dioxygen to water, using the energy produced in this reaction for translocation of protons across the mitochondrial membrane [1–3]. The pH gradient established this way across the membrane then drives the conversion of ADP to ATP [4]. Two recently determined X-ray structures of the enzyme from bovine heart [5] and *Paracoccus denitrificans* [6] at 2.8 Å resolution have shown that the catalytically active metal centers include a six-coordinate, (bis)imidazole heme *a*, an unprecedented [2Cu–2S–Cys] cluster as the $Cu_A$ site, and a dinuclear heme $a_3/Cu_B$ center. This dinuclear center is the site of dioxygen binding and reduction and contains a five-coordinate iron, with an axial imidazole and a copper ion with two [6] or three [5] histidine ligands. Despite the recent crystallographic results, several aspects of the chemistry of this prodigious molecular machine remain to be unravelled. In particular, for the dinuclear heme $a_3/Cu_B$ center, this concerns the mode of reaction with dioxygen and the correspondence between the magnetic and spectroscopic properties of the enzyme as isolated, and the X-ray structural data, since it has been widely ac-

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cepted so far that a bridging ligand between the iron(III) and copper(II) ions (absent in the structures) mediates electronic coupling between the two centers [7].

Most current models of the heme–copper center concentrated on oxidized, heterodinuclear PFe(III)–L–Cu(II) complexes, where a bridging ligand assembles two mononuclear precursors, with the aim of gaining an understanding of the magnetic and spectroscopic properties of such a peculiar heterodinuclear unit [8–10]. A different approach, that may be more convenient for reactivity studies, is based on the synthesis of porphyrin complexes modified with covalently linked chelating ligands for copper [11–15]. Our synthetic strategy has involved the attachment of a polybenzimidazole residue, as the copper ligand, to the propionic acid side chain of a natural porphyrin [14]. The initial system reported, DH-BB/Cu, was subsequently modified by the additional covalent attachment of an L-histidine residue to the second propionic acid chain of the porphyrin to give the chelated deuterohemin/copper complex DH-HBB/Cu [15]. We report here a further improvement of the model, where the L-histidine residue is replaced by the longer glycyl-L-histidine residue, which will be denoted as DH-GHBB/Cu (Fig. 1).

## 2. Results and discussion

The deuterohemin complex DH-GHBB was obtained by condensation between deuterohemin-2(18)-glycyl-L-histidine methyl ester

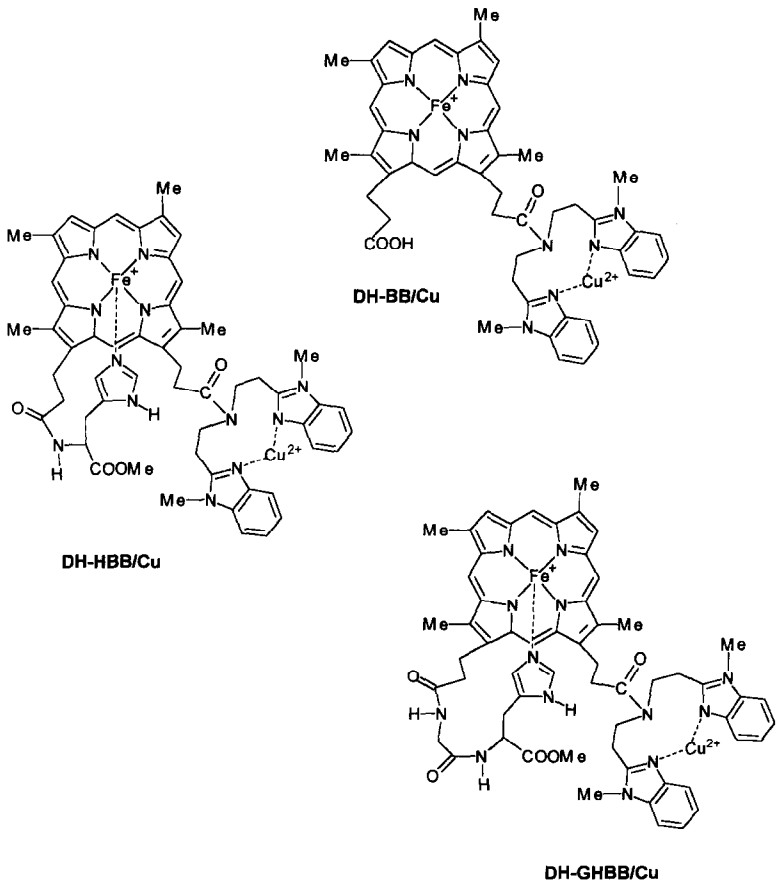


Fig. 1. Structures of the deuterohemin/copper complexes investigated.

(DH-GH) [16] and *N,N*-bis-[2-(1-methylbenzimidazol-2-yl)ethyl]amine (BB) [17]. As with the other deuterohemin complexes prepared by us, by modification of the propionic acid side chain [14–16,18–20] DH-GHBB is obtained as an equimolar mixture of the two isomers with substitution patterns 2(18) and 18(2) at the porphyrin ring positions. The main reason to proceed at the replacement of the chelating imidazole arm of the deuterohemin-L-histidine methyl ester derivative (DH-H) used previously [15] with a histidine-containing dipeptide residue stems from our recent study showing that the histidine chelate ring is strained, but that this strain is completely removed when the ring is enlarged by the introduction of a nonpolar amino acid before histidine [20]. While an L-alanyl-L-histidine dipeptide was used previously [20], solubility problems with the resulting deuterohemin derivative suggested the replacement with the more amenable glycyl-L-histidine dipeptide. The optical spectral data of the DH-GHBB complex are typical for high-spin deuterohemin complexes: the Soret, $\beta$ and $\alpha$ bands occur at 390, 485 and 590 nm, respectively, in methanol solution.

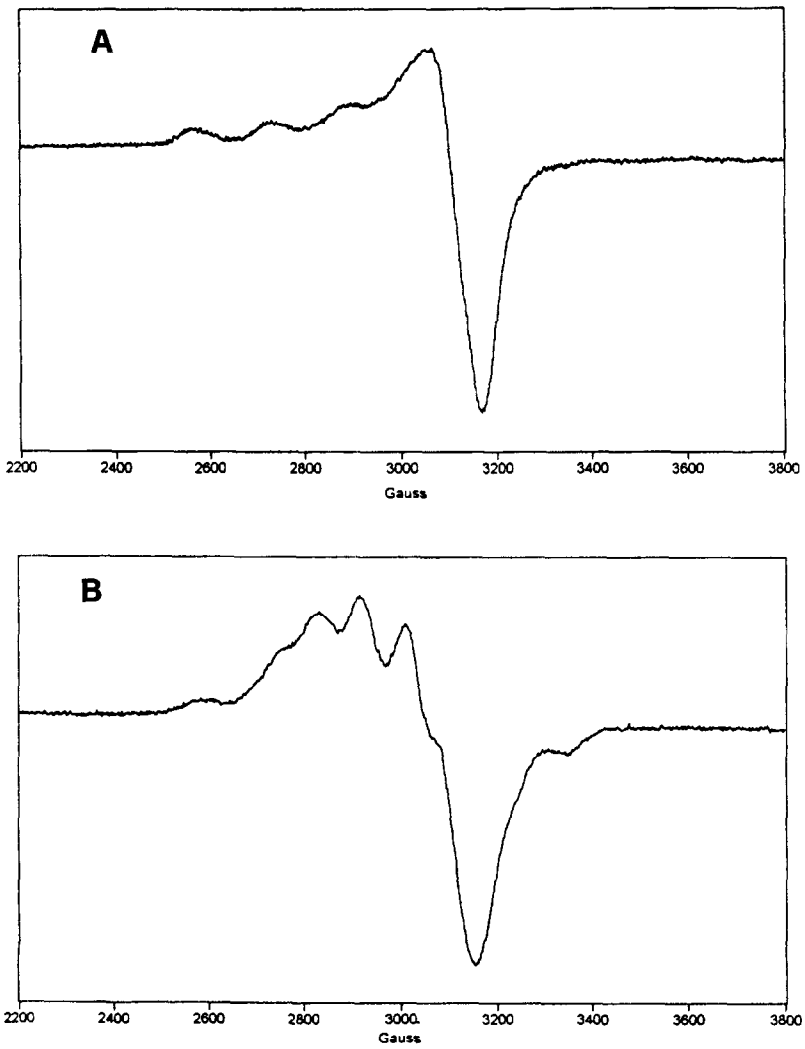


Fig. 2. EPR spectra recorded in frozen DMSO solutions at 123 K of (A) DH-HBB/Cu, and (B) DH-GHBB/Cu.

An important aspect of the coordination chemistry of the deuterohemin-bis(benzimidazole) complexes DH-BB, DH-HBB, and DH-GHBB is the observation that the presence of an imidazole group axially ligated to the iron(III) center promotes the folding of the bis(benzimidazole) arm towards the opposite part of the porphyrin plane [14,15]. This effect can be deduced by the systematically reduced affinity found for the complexes containing the BB residue for exogenous imidazole, to give the six-coordinate, low-spin adduct (Soret $\lambda_{max}$ 404 nm, visible $\lambda_{max}$ 525 nm), with respect to the corresponding deuterohemins lacking the bis(benzimidazole) arm. The data reported in Table 1 show that a similar trend is observed here for DH-GH and DH-GHBB. It is also apparent, though, that the glycyl-L-histidine side chain increases the stability of the six-coordinate adduct with respect to the corresponding complexes containing L-histidine residues, due to the stronger iron-imidazole axial bond provided by the chelated arm. The presence of a chelated imidazole residue, in any case, increases the binding constant for the exogenous, sixth imidazole ligand by one order of magnitude.

Table 1
Binding constants of imidazole to deuterohemin complexes determined by spectral titration in methanol

| Complex | $K\ M^{-1}$ | Ref. |
|---|---|---|
| DH-BB/imidazole | 350 [a] | [14] |
| DH-H | 2300 | [15] |
| DH-HBB | 1600 | [15] |
| DH-GH | 7800 | this work |
| DH-GHBB | 4300 | this work |

[a] This constant refers to the binding of the second imidazole ligand to deuterohemin-bis(benzimidazole) [14].

Binding of $Cu^{2+}$ to DH-GHBB occurs with extremely high affinity and can be followed by the small perturbation produced in the optical spectrum of DH-GHBB. Typical Cu(II) features can be observed in the EPR spectrum of the mixed DH-GHBB/Cu complex recorded in frozen solution at 123 K. Interestingly, the spectrum recorded in frozen DMSO solution appears to be rhombic and differs remarkably from that obtained for DH-HBB/Cu in the same conditions, which is characteristic of Cu(II) in tetrag-

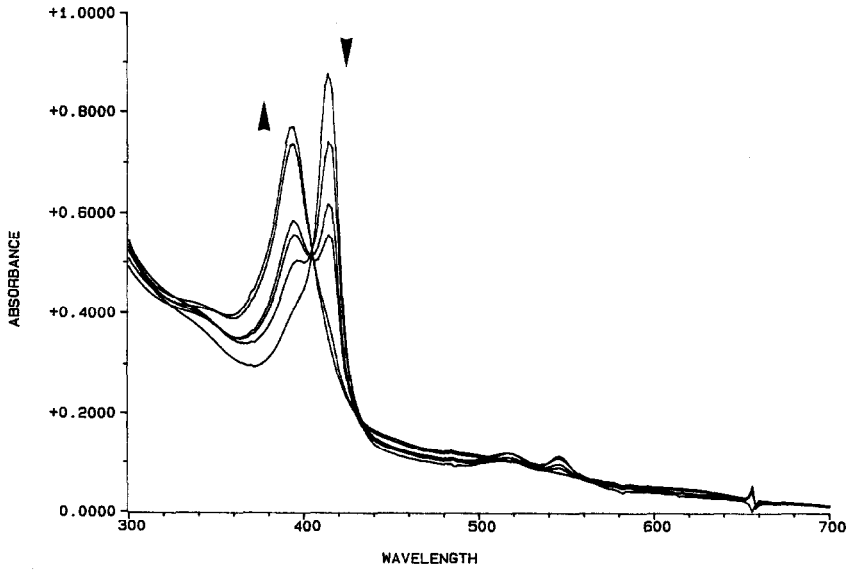


Fig. 3. UV/Vis spectra recorded during the air oxidation of the reduced Fe(II)/Cu(I) derivative of DH-GHBB/Cu in dichloromethane at room temperature.

onal symmetry (Fig. 2). The shape of the spectrum remains unchanged upon dilution of the solution, showing that the signal does not seem to be influenced by aggregation phenomena. The difference between the EPR spectra of the two heme/copper complexes may therefore be due to some significant intramolecular Fe–Cu interaction occurring in DH-GHBB/Cu that can be mediated by bridging solvent or water molecules. A weak interaction of this type seems responsible for the magnetic behavior of DH-HBB/Cu [15]; we thus expect that in DH-GHBB/Cu this effect will be stronger.

The most interesting aspect of the chemistry of the heterodinuclear complex DH-GHBB/Cu is its redox behavior. As for DH-BB/Cu and DH-HBB/Cu, anaerobic treatment of DH-GHBB/Cu with an equivalent amount of ascorbate leads to complete disappearance of the Cu(II) EPR signal, while reduction of the Fe(III) requires the addition of strong excess reducing agent. Reduction of both metal ions can be achieved using a small excess of aqueous dithionite. The slight solubility of DH-GHBB/Cu in dichloromethane allows to carry out the reduction anaerobically in a heterogenous dichloromethane/sodium dithionite mixture even without the external addition of water. Upon exposure of the reduced solution of DH-GHBB/Cu to air, the complex undergoes a rapid oxidation from the Fe(II)/Cu(I) species to the Fe(III)/Cu(II) species without any oxidative decomposition of the porphyrin at room temperature (Fig. 3). A similar behavior could be observed for DH-BB/Cu only at a rather low temperature (−45°C) [14]. For DH-HBB/Cu the nondestructive oxidative pathway prevailed at room temperature but the recovery of the initial complex was incomplete [15]. By contrast the reaction of reduced DH-BB, DH-HBB or DH-GHBB complexes with air leads to the rapid and complete bleaching of the porphyrin chromophore. We thus believe that the smooth behavior of the heme/Cu complexes with dioxygen is due to the initial formation of an intramolecular $Fe^{3+}$–$O_2^{2-}$–$Cu^{2+}$ bridge that likely evolves with the release of hydrogen peroxide and the oxidized Fe(III)/Cu(II) species. Hydrogen peroxide may in turn be reduced by a second Fe(II)/Cu(I) molecule. Our plans are to investigate the redox chemistry of DH-GHBB/Cu at low temperature, in an attempt to trap some of the reactive intermediates, and to carry out detailed magnetic investigations of the heme/copper complexes to establish the nature of the interaction between the Fe(III) and Cu(II) centers.

## 3. Experimental

Reagents and solvents were of commercially available reagent quality unless otherwise stated. Dimethylformamide (DMF) was refluxed under vacuum over BaO, stored over $CaH_2$ and distilled under reduced pressure before use. Proton NMR spectra were recorded on Bruker WP-80 or AC-200 spectrometers. Optical spectra were recorded on an HP 8452A diode-array spectrophotometer. Mass spectra were obtained with a VG 7070 EQ instrument. EPR spectra were measured in frozen solutions using a Varian E-109 spectrometer operating at X-band frequencies. The spectra of air sensitive solutions were obtained in cells fitted with Schlenk connections. Deuterohemin was prepared from hemin according to a literature method [21]. The preparation of methyl glycyl-L-histidinate dihydrochloride and deuterohemin-2(18)-glycyl-L-histidine methyl ester (DH-GH) will be reported elsewhere [16].

The preparation of the modified deuterohemin complex DH-GHBB was carried out according to the following procedure. To a solution of DH-GH (60 mg, 0.074 mmol) *N,N*-bis[2-(1-methylbenzimidazol-2-yl)ethyl]amine [17] (25 mg, 0.074 mmol), dried hydroxybenzotriazole (0.37 mmol), and triethylamine (0.37 mmol) in dry DMF (5 ml) was added HBTU (28 mg, 0.074 mmol) and the solution was stirred for 2 h at RT. The reaction mixture was then added dropwise to ice-cooled diethyl ether

(50 ml) to precipitate the deuterohemin complexes. The crude product was chromatographed on a silica gel column (4 cm × 30 cm) using a mixture of *n*-butanol–acetic acid–water (4:2:1 v/v/v). The unreacted DH-GH is eluted in this way while the product is retained by the column. Elution of DH-GHBB was performed with the use of a mixture of dichloromethane–methanol–water–triethylamine (1:2:0.2:0.5 v/v/v/v) (yield ~30%). The FABMS spectrum obtained from a nitrobenzyl alcohol matrix gave a cluster of peaks centered at the expected $m/z$ value of 1088.

Concentrations of the solutions of DH-GHBB were determined spectrophotometrically using an extinction coefficient $\varepsilon = 100000\ M^{-1}\ cm^{-1}$ for the Soret band of the compound in methanol solution. This value was determined by the pyridine hemochromogen method [21]. The mixed-metal complex DH-GHBB/Cu was obtained by mixing equimolar methanolic solutions of the modified deuterohemin and copper(II) perchlorate. The solutions were evaporated under vacuum for measurements to be carried out in different solvents.

Ligand binding experiments with deuterohemin complexes were performed spectrophotometrically by adding small amounts of concentrate solutions of the ligand to dilute solutions of the hemin. The binding constants were determined as described previously [18].

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