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Preparation and Study of Magnesium Deuteroporphyrin Myoglobin and Hemoglobin Species

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*The myoglobin and hemoglobin species containing magnesium deuteroporphyrin have been prepared and studied by electronic, circular dichroism and optical rotatory dispersion spectroscopy. The results are compared with those obtained for corresponding magnesium protoporphyrin and magnesium mesoporphyrin complexes. In all cases the magnesium–apomyoglobin species show additional band splittings. These may arise directly from differences in the protein environment or indirectly through water coordination to magnesium which is facilitated by features of the myoglobin heme pocket but inhibited in the hemoglobin complexes. The availability of results for three different porphyrins enables a red shift of spectral bands, observed in particular for MgPP-Mb**, to be specifically associated with the presence of side-chain vinyl groups.*

Introduction

The interactions of Mg porphyrin with cytochrome [1] and Mg phthalocyanine with RNA [2] have been studied in order to elucidate the properties of these high molecular weight biological environments. Recently, the interactions of other Mg complexes *e.g.* chlorophyllin, chlorophyllide and bacteriochlorophyllide with another protein, apomyoglobin, have also been intensively studied [3–7]. In order to elucidate the possible coordination state of the Mg atom, we have characterised the spectral properties for MgPP-Mb, MgPP-Hb, MgMP-Mb, MgMP-Hb [8] and Mg porphyrin in a variety of chiral amino acid solutions [9, 10].

Lately, the possibility of interactions between the peripheral substituents of the metalloporphyrin/metallochlorin and the amino acid side chains of the protein, giving rise to modulation of the physical and chemical properties of the ring system has also received considerable attention [11–14]. An effective way of probing this porphyrin–protein interaction is by modifying the structure of the heme peripheral substituent itself, and then relating these modifications to alterations in the functional properties of the protein reconstituted with these porphyrins [15]. Previous studies of this type of substitution effect, where the vinyl groups of the heme periphery are replaced by other functional groups, have produced interesting results which suggest that steric effects [15, 16], inductive effects [17, 18], and protein–heme interactions play an important role in the binding properties of hemoglobin [19, 20]. It has been suggested that in native hemoglobin, Valine FG 5 (93) is in close contact with the vinyl groups and the absence of the vinyl groups in deuteroporphyrin provides an explanation for its reduced cooperativity [21]. Quantitative calculations of the energetics of heme–protein interactions in hemoglobin, support the importance of this interaction between the vinyl group and the protein [22].

We report here the preparation and spectral properties of MgDP-Mb and MgDP-Hb. The results obtained for these species, where the vinyl groups are absent from the porphyrin rings, when compared with related MgPP and MgMP complexes enable, in particular, the role of the vinyl groups to be assessed in some detail.

Experimental

Protohaemin was prepared from whole blood by standard procedures [23, 24]. DPDME was prepared

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**Abbreviations: PP(DME) Protoporphyrin (dimethyl ester), MP(DME) Mesoporphyrin (dimethyl ester), DP(DME) Deuteroporphyrin (dimethyl ester), Mb Apomyoglobin, Hb Apohemoglobin, CMC Carboxy methyl cellulose, CD Circular dichroism, ORD Optical rotatory dispersion.

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from protohaemin according to the methods of Walter [25] and Chu and Chu [26], with minor modifications. 1 g of protohaemin was thoroughly mixed with 5 g of resorcinol and fused in an oil bath at 180–190 °C for 30 minutes. The reaction mixture was then dissolved in 98–100% formic acid and 0.5 gm of reduced iron powder added in small batches over 10 minutes. The greenish solution turned to deep violet and a sample of the solution showed no absorption band at 545 nm in pyridine–hydrazine hydrate solution [27]. Further preparative work was similar to that of Chu and Chu [26] except that purification of DPDME was carried out by using a column of Grade IV alumina. The Grade IV alumina was prepared by standing alumina in distilled water for 2 hour and dried overnight at 28 °C. The DPDME was developed with analytical grade chloroform–benzene (1:10 v/v) and the DPDME was eluted as a clean violet band. The volume of the solvent was then reduced by boiling and on cooling, long red glittering needles appeared. The purity of the DPDME was checked by the extinction coefficients [28]. The melting point of 225 °C, agrees well with the reported values of 223 °C [26] and 224.5 °C [27]. MgDPDME was prepared from DPDME and magnesium perchlorate [29, 30] and the reaction was completed when a sample of the solution showed no absorption band at 621 nm in ether solution. Base hydrolysis by NaOH gave the desired MgDP [31]. The identity of the MgDP was verified by electronic extinction coefficients [32] and thin layer chromatography whereby a single spot was obtained.

Sperm-whale met-Mb was purchased from Sigma Chemical Company. Apo-Mb was prepared by removing the heme group of met-Mb using 2-butanone [33]. Apo-Hb was prepared from ox Hb [34, 35] and used within 10 days of preparation. The purified apo-Mb and apo-Hb showed no absorption bands in the visible region.

MgDP-Mb and MgDP-Hb were prepared by the addition of a two-fold excess of standard MgDP in 0.05 *M* pyridine–water to standard apoprotein with gentle stirring. Previous studies show that Mg porphyrin binds stoichiometrically in the ratio 1:1 and 1:4 with apo-Mb and apo-Hb respectively [8]. The concentrations of apo-Hb and apo-Mb were determined by the extinction coefficients of 16.2 m*M* cm^{-1} [36] and 15.8 m*M* cm^{-1} [37] at 280 nm respectively. Within half an hour, the mixture was placed on a long Sephadex column, which was previously equilibrated with 10 m*M* phosphate solution, to remove the pyridine and excess MgDP. Sephadex G 50-80 and Sephadex G 75-120 were used for purification of the reconstituted MgDP–Mb and MgDP-Hb respectively. The reconstituted complex was then passed through a CMC 32 column of pH = 7 to remove any MgDP that was loosely bound to the surface of the protein. The MgDP-globin complex was developed as a clean pink band with 10 m*M* phosphate solution. The reconstituted material was then further dialysed against 10 m*M* phosphate (pH = 6.86). Using Sephadex G 75-120 for MgDP-Hb which has the capability to exclude proteins of molecular weight greater than 50,000 daltons, it was not necessary to check the formation of the tetramer unit by the flow rate method [38]. Evidence that only one reconstituted product is formed in all cases is obtained by using the procedure of Srivastava [39]. The MgDP-globin complex was passed through a long Sephadex column followed by a long CMC 32 column. Each fraction was checked by electronic absorption spectroscopy which strongly suggests the formation of only one protein complex. All precipitate formed was removed either by filtering or centrifuging.

Electronic absorption spectra were recorded on a Varian Superscan 3 UV–visible spectrophotometer. Analytical grade chloroform, pyridine and benzene were purchased from British Drug Houses Ltd and stored in the dark at 0 °C over 4 Å molecular sieves under nitrogen. Other reagents used were of analytical grade.

ORD and CD measurements were recorded on a Jasco ORD UV–5 spectrophotometer. Reagents were kept away from strong lights and all apparatus was covered by aluminium foil. All the preparative work was carried out at temperatures between 0 °C and 4 °C. All measurements were recorded at room temperature unless mentioned otherwise.

Results

Spectra of MgDP-Mb and MgDP-Hb Species

Electronic spectra

The absorption maxima wavelengths of MgDP-Hb are similar to those of MgDP in ether, although the relative intensities of the Q_1 and Q_o bands are higher in the former case (Table I). The Q_o transition for MgDP-Mb is split into two bands of unequal intensities (Fig. 2), as observed also for MgMP-Mb and MgPP-Mb. The position of the major Q_o band of MgDP-Mb (582 nm) is red-shifted with respect to the Q_o absorption for MgDP-Hb (578 nm). This red shift of 4 nm is significantly smaller than the shift of 9 nm observed for MgPP-Mb relative to MgPP-Hb and about the same as the red shift for the MgMP species (see bands labelled b in Table I).

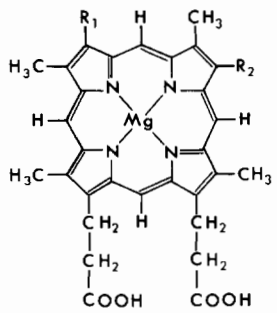
CD spectra

MgDP does not show any definite CD spectra in pyridine, ether or benzene. The reconstituted MgDP-Mb and MgDP-Hb show dominant Cotton effects for all the absorption bands.

TABLE I. Comparison of the Electronic and Circular Dichroism Data for the Various Metal Porphyrin Complexes.

Compound	Electronic Bands (nm)						Circular Dichroism Bands (nm)			Reference
	Soret		Q_1		Q_o		Soret	Q_1	Q_o	
	λ_{max}		λ_{max}	A	λ_{max}	A	λ_{max}	λ_{max}	λ_{max}	
MgPP in ether	417	1.000	550	0.060	589	0.060				[8]
MgPP-Hb	420	1.000	550	0.070	589	0.060	425	548, 558	589	[8]
MgPP-Mb	424	1.000	553 560(sh) 545(sh)	0.080 0.064 0.044	598[b] 590(sh)	0.065 0.039	420	538–552, 560	600	[8]
MgMP in ether	409	1.000	544	0.041	581	0.041				[8]
MgMP-Hb	408	1.000	543	0.053	580.5	0.040	412	542, 549	576	[8]
MgMP-Mb	409	1.000	542 549(sh) 533(sh)	0.063 0.050 0.029	585[b] 574	0.040 0.025	408	542, 550	574, 585	[8]
MgDP in ether	405	1.000	542	0.040	578.5	0.03				this work
MgDP-Hb	406	1.000	541.5	0.049	578	0.03	408	533, 547	574	this work
MgDP-Mb	408	1.000	542 548(sh) 533(sh)	0.061 0.049 0.040	582[b] 572(sh)	0.027 0.023	406	533, 549	576	this work

[a]Absorbance data normalised to 1.000 relative to most intense transition. [b]Major component (which is red-shifted with respect to the corresponding Hb band – see text); sh = shoulder.



Compound	R_1	R_2
Mg Protoporphyrin	$CH=CH_2$	$CH=CH_2$
Mg Mesoporphyrin	CH_2-CH_3	CH_2-CH_3
Mg Deuteroporphyrin	H	H

Fig. 1. Structure and nomenclature for magnesium porphyrins discussed in text.

The Q_o transition for MgDP-Hb has a single band while the Q_1 transition is split into two bands (Fig. 3). The Soret band for MgDP-Hb is split unsymmetrically into a dominant positive band and a small negative band at 395 nm. The Q_o and Q_1 transitions (at *ca.* 548 nm) of MgDP-Mb are shifted by about 2 nm to the red region (for both transitions) relative to those for MgDP-Hb. The band at 533 nm remains the same for both MgDP-Mb and MgDP-Hb. However, the Soret band for MgDP-Mb is blue-shifted by 2 nm and there is no negative region.

ORD spectra

MgDP-Hb has peaks at 582, 550, 413 and 396 nm and troughs at 576, 543, 533, 401 and 393 nm. MgDP-Mb has peaks at 586, 554, 534, 410 and troughs at 578, 544, 526 and 400 nm. The maximum peaks of the CD spectra of MgDP-Hb and MgDP-Mb correspond very closely to the zero rotation of the corresponding ORD spectra.

Discussion

The preparative results for MgDP-Mb and MgDP-Hb, together with the close similarity of electronic and CD spectra of these complexes with those for corresponding PP compounds (for which binding ratios were established [8]), show that well defined 1:1 and 1:4 Mb and Hb complexes are also formed with MgDP. This contrasts with results for the chlorophyllide–apomyoglobin complexes where three different species with different electronic and CD spectral features were obtained [7].

The spectral features of MgDP-Mb, in particular the splitting of the Q_o transition, closely parallel those reported for MgPP-Mb and MgMP-Mb [8]. Thus it is concluded, for the same reasons as those presented in ref. 8 for the PP and MP complexes, that MgDP-Mb is also six-coordinate. Similarly it

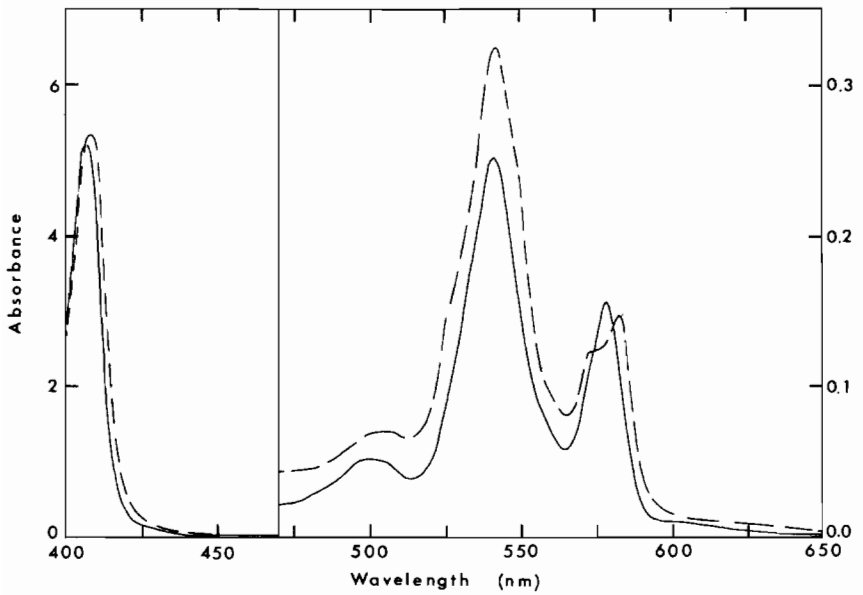


Fig. 2. Electronic absorption spectra of MgDP-Hb (———) and MgDP-Mb (-------) in 10 m*M* phosphate buffer of pH = 7.0.

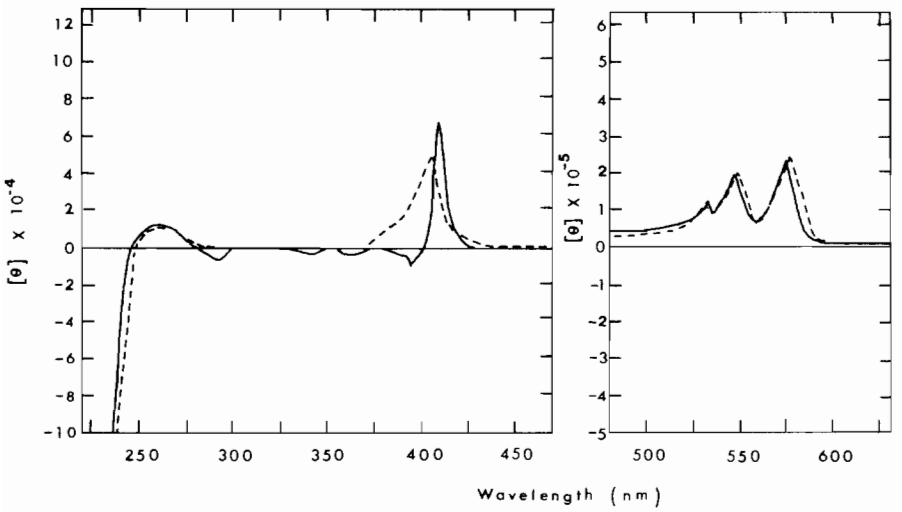


Fig. 3. Circular dichroism spectra of MgDP-Hb (———) and MgDP-Mb (-------) in 10 m*M* phosphate buffer of pH = 7.0 redrawn to show main bands. Region from 450 to 250 nm was diluted 20 × relative to the region between 500 and 620 nm.

can be concluded that MgDP-Hb, like MgPP-Hb and MgMP-Hb, contains five-coordinated magnesium entities. It may be noted that by contrast with these magnesium species, Zn porphyrin-Mb and -Hb give essentially the same electronic spectra [40–42]. This may be due to the lower tendency of zinc to form six-coordinate species of this type.

The main result for the DP complexes reported here is the magnitude of a red shift of the Q_o transition in going from MgDP-Hb to MgDP-Mb. Although relatively small in magnitude (4 nm), a comparison of this value with corresponding ones for the PP and MP analogues (9 and 4.5 nm respectively) provides some measure of the effects of porphyrin substituents on the electronic transitions.

A change in coordination number may account for part of the red shift but the difference for MgDP and MgPP complexes (4 nm compared with 9 nm) suggests other factors are involved. The order of the red shift, MgPP > MgDP ≅ MgMP, is similar to that observed in iron [43–45] and cobalt [38] complexes. The magnitudes of these red-shifts for the oxy protein complexes of FePP, FeDP and FeMP are 4, 1 and 1 nm respectively [43–45]. Similar

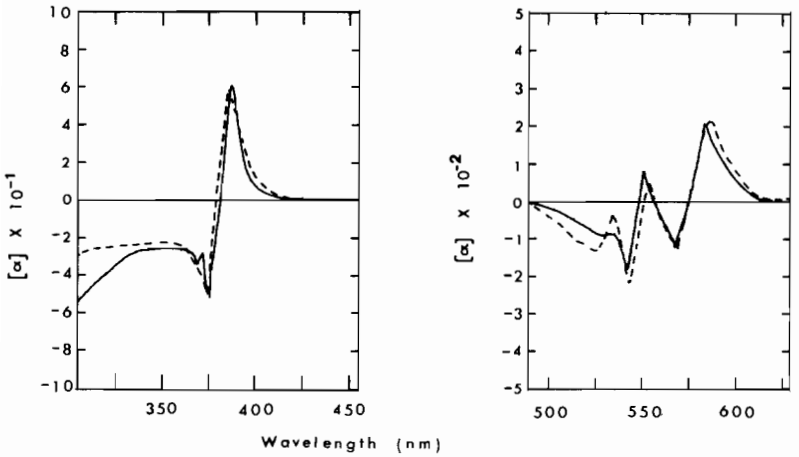


Fig. 4. Optical rotatory dispersion of MgDP-Hb (———) and MgDP-Mb (-------) under pH = 7 redrawn to show main bands.

magnitudes of red-shifts are also observed for the iron protein complexes of isospirographis, spirographis and 2,4-diformyl [46]. For oxy CoPP–protein complexes, this red-shift is about 6 nm, a further indication of the association of some particular effect with PP.

Various factors can possibly contribute to the Q_o red-shift. The first factor involves deprotonation of the proximal imidazole. Mincey and Traylor have shown that different imidazolate axial ligands red shift the absorption bands by different magnitudes [47]. However, in the case of MgPP-Mb, this factor does not appear to contribute significantly to the red-shift. Since all the experiments were performed at pH 7, the degree of deprotonation of the proximal imidazole of apo-Mb should remain the same for MgDP-Mb, MgMP-Mb and MgPP-Mb. Consequently any red shift associated with this effect should be approximately the same for all three complexes.

The second factor involves changes in the polarity of the heme environments for hemoglobin and myoglobin [48]. Romberg and Kassner, using model heme complexes in a variety of solvents have shown that the red-shift correlates with the polarity of the solvents [49]. The changes in the polarity of the heme environment from MgPP-Mb to MgPP-Hb is likely to be similar to that from MgDP-Mb to MgDP-Hb. But only a small red-shift was observed when the electronic spectra of MgDP-Mb and MgDP-Hb were compared. Thus, this factor is unlikely to contribute significantly to the larger red-shift observed for MgPP.

The third factor involves conformational changes affecting porphyrin protein interactions. However, the similarities between the shapes of the CD spectra of MgPP-Mb and MgDP-Mb suggest that there is no difference in the orientation of MgPP and MgDP in myoglobin. Similar results were observed for the iron myoglobin analogues [50]. Furthermore, the Soret bands of MgDP-Mb and MgPP-Mb suggest that the polarisation direction is the same and occurs along the bridging methine carbons in both protein complexes [51]. In addition, Perutz and coworkers have shown that changes in the T → R states of six-coordinated iron complexes changes the electronic spectra by only 0.5 nm to 2 nm [52]. Thus, this factor cannot be considered to contribute significantly to the red-shift.

The fourth factor is different interactions between the 2,4 disubstitutents and the protein environments of hemoglobin and myoglobin. X-ray [53, 54] and other studies have shown that the heme pocket of myoglobin is more rigid than in hemoglobin [55] and leghemoglobin [56]. Thus, the peripheral substituents of the Mg porphyrin are more likely to interact with the side chains of the amino acid groups in myoglobin than in hemoglobin. Evidence for the importance of interaction between the protein and the peripheral substituents has been obtained by Sono and Asakura [46], where the oxy protein complexes of the two isomers, spirographis and isospirographis, show different absorption spectra. In the case of MgMP-Mb, we proposed earlier that steric interaction between the ethyl groups of MgMP and the protein affects the nature of the six-coordination geometry and hence the magnitude of the red-shift [8]. However, the similarity now found for the shapes of the CD spectra of MgDP-Mb and MgPP-Mb does not support that interpretation. Rather it appears that a particular differential electronic effect is associated with the 2,4 vinyl substituents of MgPP.

NMR studies using ^{13}CO and various 2,4 disubstituted iron myoglobins and hemoglobins support this idea that the electronic interactions between the 2,4 disubstituents and the two heme environments are

different [57]. Recent experimental results from iron and cobalt protein analogues suggest that both steric [15, 16] and electronic [17, 18] interactions are important. The vinyl groups, being electron withdrawing, can transfer charge to the side-chains of the amino acid groups either by direct charge transfer [58] or by hydrogen bonding [11–12]. Other recent studies show that the positions of the electronic bands are indeed affected when there are electronic interactions between the vinyl groups and another charged species *e.g.* Cu(I) [59]. Thus overall, we conclude that, while various factors may contribute to the red-shift of the Q_o band in going from Hb to Mb complexes, a particular electronic effect produces an additional red-shift for MgPP. This may be envisaged to arise from differences in an electronic interaction between the vinyl groups and the protein environments of hemoglobin and myoglobin respectively.

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