What Is the Crucial Factor for Vibrational Circular Dichroism in Hemoprotein Ligands?

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Abstract: Intense vibrational circular dichroism (VCD) bands for the azide antisymmetric stretching vibration in the azide complex of a C_2-chiral strapped iron porphyrin have been observed with anisotropy values of ca. 2×10^{-3}. This is the first case in which the extremely strong VCD band has been measured for a ligand vibration of iron porphyrin without an apo-protein. The VCD spectrum of myoglobin azide shortly after reconstitution with its own heme exhibited a weak band but over time the intensity with negative sign gradually recovered. The combination of these two results suggests that the chiral environment produced by the peripheral substituents on the porphyrin ring, which give rise to a diastereotopic plane, is responsible for the generation of the intense VCD in hemoproteins whereas specific interactions of distal residues with the porphyrin ring and the ligands in halo-proteins are not always necessary.

Introduction

The distal amino acid residues in myoglobin (Mb) and hemoglobin (Hb) are elaborately designed to control the chemical reaction of ligands to the heme iron. For instance, the formation of a hydrogen bond between the His-E7 (distal histidine) and the oxygen ligand, first proved by neutron diffraction studies,[1] stabilizes the oxyform of Mb[2] and steric hindrance by the Valine-E11 is critical for tuning the ligand affinity in the β subunit of Hb.[3] Extensive resonance Raman and IR studies[4] have revealed that the distal His also acts as an obstacle for linear ligands such as carbon monoxide. However, our understanding of reaction mechanisms in hemoproteins is still incomplete and further advances in theory or a new methodology are necessary.

Recently, two VCD studies of ligands bound to hemoproteins have been published,[5,6] the results of which give valuable new insights into the electronic states as well as the coordination geometry of hemoproteins. Bormett et al.[5] observed a reduction in the intensity of the VCD signal of heme−azide complexes for mutant myoglobins in which His-E7 was replaced with glycine and Val-E11 was replaced with asparagine. These results were used to demonstrate that the VCD depends upon subtle interactions of the azide ligand with the distal heme pocket residues. We had earlier suggested that the asymmetry of the porphyrin ring may play an important role in generating the intense VCD anisotropy based on the result[6] that no VCD band for the metMb(OEP)N_3 reconstituted with Fe(OEP)Cl (OEP = octaethylporphyrin) was observed.[7]

In this study the VCD spectra of an azide complex in the iron porphyrin derived from the antipodes of a C_2-chiral strapped porphyrin[8] were investigated in order to elucidate the role of the distal residues in metMbN_3 in the generation of the intense VCD band associated with the azide antisymmetric stretching vibration. The time evolution of the VCD band was also examined for the azide ligand in metMbN_3 after reconstitution of Mb with its own heme.

The results in the present study indicate that a diastereotopic plane due to the order of peripheral substituents on the porphyrin ring is the crucial factor for the generation of the intense VCD band of ligands in hemoproteins but so-called specific interactions of distal amino acids with the azide ligand are not necessary.

Experimental Section

Iron Complex of C_2-Chiral Strapped Porphyrin. Figure 1 depicts the structural scheme of the iron porphyrin derived from the antipodes of a C_2-chiral 1,4-xylylene-strapped porphyrin (the porphyrin was derived from dihexyldeuteroporphyrin 2 dimethyl ester) which was generously gifted from Drs. K. Konishi and T. Aida of the University of Tokyo. The method of synthesis and optical resolution of the metal complexes are described elsewhere,[8] but the absolute configuration has not been determined. The VCD spectra were measured for the azide complex of an antipode ((+)-FeSP) which exhibits a positive Cotton effect at ca. 350 and 400 nm in the electronic CD spectrum. The azide complex of (+)-FeSP with 1-methylimidazole (1-MeIm) was prepared

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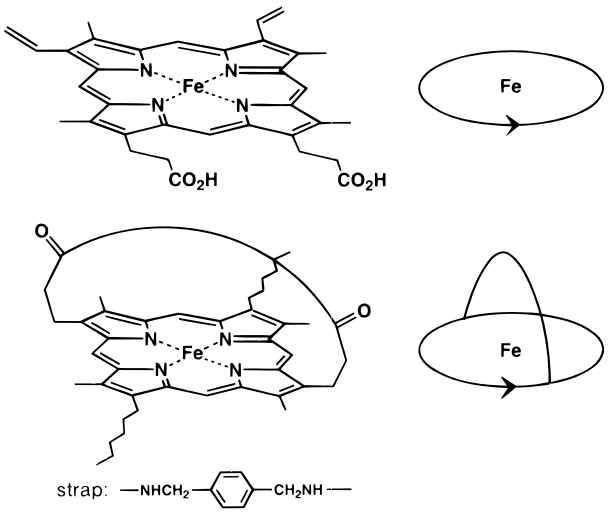


Figure 1. Molecular structures of protoporphyrin IX Fe(PPIX) and strapped porphyrin (+)-FeSP. The molecular structure of (+)-FeSP depicted here is tentative since the absolute configurations have not been determined yet. The arrows are defined for arrangements of peripheral substituents in the order of methyl, vinyl, methyl, vinyl, methyl, propionyl, propionyl, and methyl groups in Fe(PPIX) and methyl, hexyl, methyl, *p*-xylylenyl (strap), methyl, hexyl, methyl, and *p*-xylylenyl (strap) groups in (+)-FeSP.

by adding a 20-fold excess of NaN_3 and a 50-fold excess of 1-MeIm to a 4.5 mM dimethyl sulfoxide (DMSO) solution of (+)-FeSP. The azide complex of an iron protoporphyrin IX chloride, Fe(PPIX)Cl, was prepared by adding equimolar NaN_3 and a 2-fold or a 15-fold excess of 1-MeIm to 23 mM DMSO solution of Fe(PPIX)Cl. NaN_3, 1-MeIm, and DMSO were purchased from Wako Pure Chemicals and used without further purification.

Reconstitution of Mb. The reconstitution of horse skeletal muscle Mb (purchased from Sigma) with Fe(PPIX)Cl (Sigma) was carried out by Teale's method.[9] The reconstituted Mb was purified on a DEAE Cellulose (Wako Pure Chemical Industries) column. A 5-fold excess of NaN_3 was added to the reconstituted Mb after 60 min. The metMbN_3 was concentrated up to 5.6 mM by ultrafiltration (Minimodule NM-3, Asahi Kasei) and the first measurement of the VCD spectrum was started 440 min after the reconstitution. Five sets of data were acquired from 440 to 860 min after reconstitution.

VCD and IR Measurements. The IR absorption and VCD spectra in Figures 2 and 4 were measured on a JASCO J-200E dispersive VCD instrument with 20 cm^{-1} resolution, time constant 1 s, scanning speed 100 cm^{-1}/min, and 50 accumulations. The instrumentation has been described in detail elsewhere.[10] The IR absorption spectra in Figure 3 were recorded on a JASCO FT-700 instrument with 1 cm^{-1} resolution and 100 accumulations. All spectra were measured in DMSO solution, except for Mb in aqueous solution, using a CaF_2 cell of 50 mm path length. The anisotropy ratio for the azide complex of (+)-FeSP was determined from the integrated intensity.

Results and Discussion

VCD Band of Azide Bound to Heme Iron without Apo-Protein. Figure 2 illustrates the VCD and IR absorption spectra of (+)-FeSP with 1-MeIm and azide ligands in the frequency region of the azide antisymmetric stretching vibration. An intense positive VCD band is observed at 2000 cm^{-1} with an estimated $|g|$ value of at least 10^{-3}.[11] In addition, a broad negative VCD band is also detected at about 2020 cm^{-1}, which may be associated with the broad asymmetric feature on the

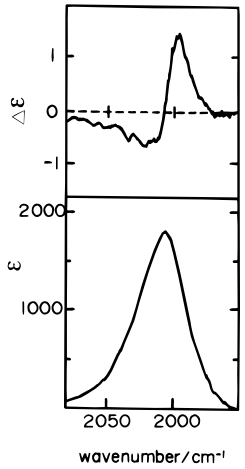
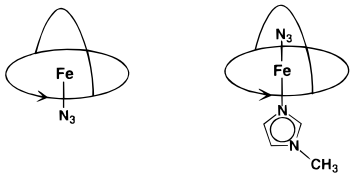


Figure 2. IR absorption and VCD (50 scans) spectra of 4.5 mM (+)-FeSP with 82.2 mM NaN_3 and 226.1 mM 1-MeIm.

high wavenumber side of the IR absorption band. It should be noted that this is the first observation of strong VCD in a complex with the iron porphyrin without apo-protein. This suggests that both the strap in this complex and also the apo-protein in metMbN_3 play a similar role in the generation of the intense VCD.

The 1-MeIm ligand can only bind to (+)-FeSP on the space opposite the strap (with a binding constant of 4.3×10^2 dm^3 mol^{-1} at 25 °C in CH_2Cl_2) since this ligand is too bulky to bind on the same side as the strap.[8b] In contrast, an azide ligand is able to bind to either face of (+)-FeSP since the strap is long enough to accommodate an azide ligand. Therefore, it is deduced that the two species, with azide coordinated at the open space (**1**) and at the strapped space (**2**), may coexist under our experimental conditions. Although DMSO molecules are



1 **2**

known to be able to ligate to iron porphyrins,[12,13] they may not coordinate to the iron at the strapped space in **1** for steric reasons. In addition, it is unlikely that a solvent DMSO molecule coordinates to the iron in the open space instead of 1-MeIm in **2** because 1-MeIm has a much stronger binding affinity than DMSO.[14] The arrow on the porphyrin ring indicates that the chiral strapped porphyrin has diastereotopic faces as shown in Figure 1.

The equilibrium between the high spin and the low spin iron states in metMbN_3 has been investigated by magnetic susceptibility measurements[15,16] and it was proved that near room temperature there is a spin equilibrium with the low spin state being more stable than the high spin state. McCoy and Caughey[17] first assigned IR absorption bands at 2046 and 2023 cm^{-1} to the azide vibrations in high spin and low spin forms of

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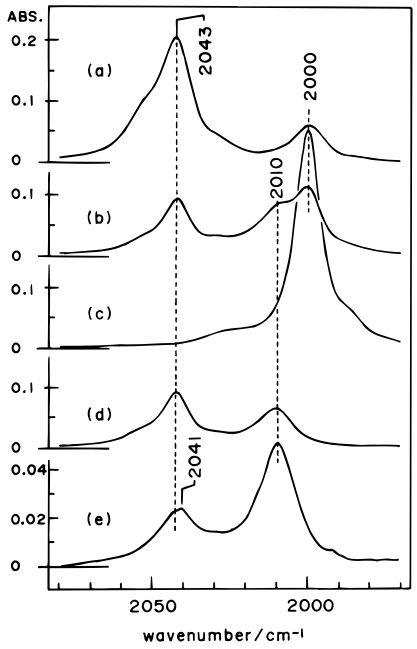
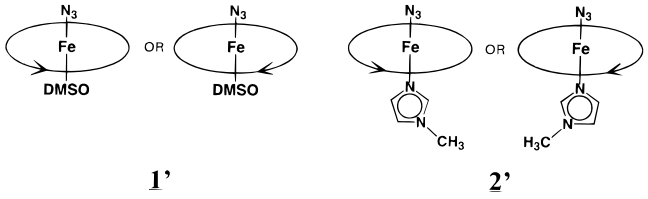


Figure 3. IR absorption spectra of the azide stretching vibrations in DMSO solutions (path length 58 mm). (a) 22.9 mM Fe(PPIX)Cl with 26.0 mM NaN_3, (b) 22.9 mM Fe(PPIX)Cl with 26.0 mM NaN_3 and 48 mM 1-MeIm, (c) 24.6 mM NaN_3, (d) difference spectrum (b $-$ 0.27 $\times$ c), and (e) (22.9 mM Fe(PPIX)Cl with 26.0 mM NaN_3 and 365 mM 1-MeIm) $-$ 0.63 $\times$ c.

metMbN_3, respectively. Alben and Fajer[18] demonstrated that the high spin iron was bound to N_3^- ionically and to the low spin iron covalently. Consequently, the hexacoordinated complex **2** could be in spin equilibrium if the situation for metMbN_3 were applied to this model system. Two IR bands were also observed for the model complexes of azide hemoproteins such as Fe(PPIX)(N_3)(DMSO),[19] Fe(PPIX)(N_3)(1-MeIm)[14], and Fe(PPIX)(N_3)(2-MeIm).[14] The bands at 2042 and 2010 cm^{-1} for Fe(PPIX)(N_3)(1-MeIm), for instance, were assigned to the vibrations of azide coordinated in the high spin and low spin forms, respectively, as demonstrated in metMbN_3. Therefore, at most three distinct chemical species, **1**, high-spin **2**, and low-spin **2**, contribute to the spectral pattern shown in Figure 2.

Figure 3 displays the effect of a trans-ligand on the stretching frequency of azide bound to the iron porphyrin. Figure 3a shows the IR absorption spectrum of Fe(PPIX)Cl with equimolar NaN_3 in DMSO. Three absorption bands were observed at 2055 (shoulder), 2043, and 2000 cm^{-1}. When 2-fold equimolar 1-MeIm was added to (a), a weak band appeared at 2010 cm^{-1} while the band intensity at 2043 cm^{-1} decreased as shown in Figure 3b. Figure 3d, the difference spectrum of b $-$ NaN_3 (c), clearly shows two absorption bands at 2043 and 2010 cm^{-1}. Therefore the band at 2043 cm^{-1} is attributable to the form **1′** and the band at 2010 cm^{-1} is due to the form **2′**. Similar spectra were obtained for imidazole and 4-methylimidazole (data not shown). As the relative intensity at 2010 cm^{-1} increases with addition of a large excess of 1-MeIm, the band at 2043 cm^{-1} shifts slightly down to produce a new band at 2041 cm^{-1} as shown in Figure 3e. Neya et al.[14] have demonstrated that the relative intensity of the bands ($\sim$2042/2010) show a temperature dependence due to the spin equilibrium under similar conditions. Therefore, the band at 2041 cm^{-1} is assigned to the azide stretching vibrations in the high spin complex and the band at 2010 cm^{-1} to the low spin species with 1-MeIm (**2′**) as the 6th ligand. Here, the arrow on the porphyrin ring signifies that the porphyrin has enantiotopic faces. Consequently, both **1′** and **2′** exist in racemic mixtures so that no VCD bands are expected.



1′ **2′**

It was thought that only the low-spin complexes show a VCD band probably due to an efficient overlap between the Π^* molecular orbitals of the ligand and the porphyrin ring through the iron d_π orbitals.[6] This suggests that the low-spin species of **2** gives rise to the VCD band. We could assign negative and positive VCD bands in Figure 2 to the azide vibrations of **1** and low spin **2**, respectively, from comparison of the spectra in Figure 2 with the absorption bands in Figure 3d corresponding to structures **1′** and **2′**. The half-band-width of the positive VCD band is narrower to some extent than that of the negative one, which is consistent with the more ordered nature of the azide ligand wrapped by the strap(**2**).[17,20]

In conclusion, the presence of the diastereotopic faces on the porphyrin ring seems to be one of the important factors in the generation of the intense VCD band in the heme system. Neverthless, it is still not clear how the strap in (+)FeSP and the apo-protein in metMbN_3 contribute to the strong VCD signal. This matter will be resolved after the assessment of the reconstitution experiment described in the following section.

Time Evolution of VCD of the Azide in Reconstituted metMbN_3. The time evolution of VCD of the azide ligand in reconstituted metMbN_3 was measured. Figure 4 shows the time dependence of the VCD spectra of metMbN_3 after reconstitution with hemin as described in the Experimental Section. The VCD spectra of A, B, C, D and E represent 440, 560, 650, 740, and 860 min, respectively, after reconstitution. The negative azide VCD band intensity is very weak 440 min after reconstitution but the intensity of this band gradually recovers with time.

La Mar et al.[21] have demonstrated that the initial product upon reacting apoMb with hemin is the halo-protein with the porphyrin 1:1 rotationally disordered about the axis bisecting the two propionic substituents (the native form called **H**ead and the reverse form **T**ail). Olson et al.[22] showed that freshly reconstituted Mb exhibited considerably less CD in the Soret wavelength region than the native protein. Santucci et al.[23] reported that the CD intensity of MbCO reconstituted with a symmetrical heme was much lower than that with a natural heme and remained constant with time. The rate of equilibrium of the disordered state was also determined using proton NMR[21] and CD spectroscopy.[22, 24]

The two azide complexes, described as **H** and **T** correspond to two enantiomorphic forms, depicted as **2′** (left) and **2′** (right), respectively, if only the active site in Mb is focused on. Therefore, the VCD observations may be qualitatively interpreted as follows. The VCD intensity could be zero im-

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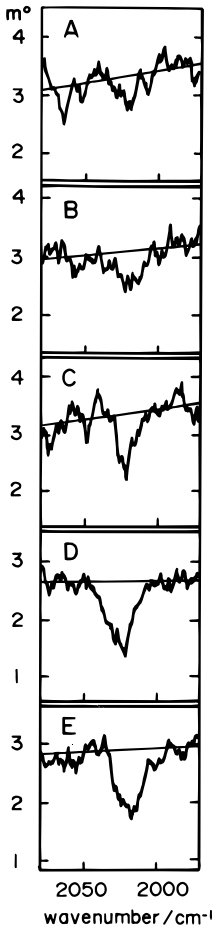

Figure 4. The time dependence of VCD spectra of 5.6 mM metMbN_3 at pH 7.0 at various intervals of time after the reconstitution with Fe(PPIX)Cl. (A) 440, (B) 560, (C) 650, (D) 740, and (E) 860 min.

mediately after reconstitution if the concentration of **H** was equal to that of **T**. However, the relaxation of the heme ring to the native form **H**, according to the rule of intramolecular reorientation, causes the growth of negative VCD band with time. Unfortunately, the poor S/N of the data precluded simulation of a reversible unimolecular reaction.

The two azide complexes described as **H** and **T** also correspond to **1** and **2**, respectively, from the standpoint of VCD, that is, the VCD spectra of **H** and **1** exhibit strong bands with negative sign while those of **T** and **2** exhibit positive bands. The only difference between them seems to be that the antisymmetric stretching frequencies of the azide ligands coincide with each other for **H** and **T** but not for **1** and **2**, which results in a near cancelling of the VCD band shortly after reconstitution and then the recovery of the negative VCD band with time and also results in the observation of the intense VCD bands with negative and positive sign in (+)FeSP. Therefore it may be concluded that both the strap in (+)FeSP and the apo-protein in met MbN_3 are essential only for generating the diastereotopic faces on the porphyrin rings.

VCD Intensity for the Azide Ligand in metMbN_3. This study has demonstrated that the azide complex of a C_2-chiral strapped iron porphyrin shows extraordinarily intense VCD bands with negative sign for structure **1** and positive sign for **2**. The time dependence of VCD of reconstituted metMbN_3 has shown that only a weak band is observed shortly after reconstitution but that the negative VCD band gradually recovers over time.

The VCD with opposite sign for structures **1** and **2** of the strapped porphyrin complex could well be explained in terms of the diastereotopic plane formed by the C_2-symmetrically arranged peripheral substituents of methyl, hexyl, methyl, and xylylenyl groups which produce the opposite chiral environments for the azides in the form of **1** and **2**. The asymmetrically arranged peripheral substituents (methyl, vinyl, methyl, vinyl, methyl, propionyl, propionyl, methyl) on the porphyrin in metMbN_3 also give rise to an diastereotopic plane which produces the opposite chiral environment for the azide coordinated in the **H** and **T** forms. The azide in metMb(OEP)N_3 reconstituted with the symmetric octaethylporphyrin, which has neither enantiotopic nor diastereotopic planes, does not exhibit any detectable VCD.[6] These results suggest that the chiral environment produced by the asymmetrically arranged peripheral substituents on the porphyrin ring play an important role in generating the intense VCD for ligands in hemoproteins. An intriguing result has been reported that metMbN_3 reconstituted with deuteroheme (in which there is no vinyl group) showed the same anisotropy value as the native metMbN_3,[5] which is inconsistent with the conjecture of vinyl group involvement in the VCD[25] but consistent with our proposal mentioned above because we just take notice of the asymmetrical arrangment of peripheral substituents. Bormett et al.[5] have also demonstrated that the VCD spectra of heme−azide complexes depend upon subtle interactions of the azide ligand with the distal residues by the observation of the reduction of the VCD signal for mutant Mb. As regards the reduction of the VCD signal, it is necessary to take account of the coexistence of a small amount of the stable **T** form as well as the dominant **H** form in the mutant Mb if our argument is acceptable.

The present study has, for the first time, shown that strong VCD bands for the azide complex of the strapped porphyrin are observed without apo-protein. The results together with the reconstitution study indicate that some specific interactions between the ligands and distal residues in the protein would not always be necessary for the generation of strong VCD of the ligands in hemoproteins. Further studies on the relationships of substituents between protoporphyrin IX and the strapped porphyrin and also on how substituents contribute to generating the magnetic transition dipole moment are currently in progress.

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