# Resonance Raman Spectra of Cobalt Myoglobins and Cobalt Porphyrins. Evaluation of Protein Effects on Porphyrin Structure

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**Abstract:** Resonance Raman spectra have been obtained of oxy and deoxy cobalt myoglobins containing cobalt protoporphyrin IX, deuteroporphyrin IX, and mesoporphyrin IX as the prosthetic groups. Spectra of the same three cobalt porphyrins, in both the (II) and (III) oxidation states, have been obtained in piperidine solution and in solutions of other organic nitrogen bases. The results imply that tension imposed upon the cobalt heme by the globin results in little distortion of the metal–porphyrin moiety from its unconstrained geometry.

Cobalt-substituted hemoglobins (CoHb) and myoglobins (CoMb) possess properties which closely parallel those of their iron-containing analogs.[2-6] First prepared by Hoffman and Petering,[2a] these "coboglobins" bind oxygen reversibly and, in the case of CoHb, cooperatively, with retention of the heterotropic linkage properties of native hemoglobin.[3,4] In view of the proposal by Perutz[7] that displacement (~0.75 Å) of the iron atom into the heme plane upon oxygenation triggers cooperativity in hemoglobin, and the observed cooperativity and heterotropic properties of CoHb, the question of the stereochemistry of the cobalt heme moiety in coboglobins is of some importance. Therefore there has been considerable recent interest in the structure[8-18] and axial ligation (including oxygenation)[19-26] of cobalt porphyrins and related complexes.[27-29] Structural data indicate that the maximum displacement of an imidazole-type axial ligand relative to the mean porphyrin plane, between five-coordinate cobalt(II) and six-coordinate cobalt(III) porphyrins, is about 0.47 Å in the absence of significant external constraints.[13,15,17,18] However, the question of the influence of strain imposed by the apoprotein upon the structure of the unconstrained porphyrin remains.[14,15,18,30,31]

Resonance-enhanced Raman spectroscopy has been successfully applied to several heme proteins, including hemoglobin derivatives.[32-37] Examination of these spectra has led to the identification of "indicator bands", whose frequencies are particularly sensitive to the oxidation state of the iron atom and/or its position with respect to the mean heme plane.[38] In a previous study[39] we reported the resonance Raman spectra of oxy- and deoxy-CoHb, and inferred that apohemoglobin has little effect upon the displacement of the cobalt atom from the mean heme plane in deoxy-CoHb compared to $Co^{II}PP$. We now report a more comprehensive study of the effect of apomyoglobin upon the resonance Raman spectra of three cobalt porphyrins: cobalt protoporphyrin IX, deuteroporphyrin IX (CoDP), and mesoporphyrin IX (CoMP) (see Figure 1). The data are discussed in terms of possible steric constraints imposed upon these cobalt hemes by the apoprotein.

## Experimental Section

The preparative methods employed for cobalt porphyrins and cobalt myoglobins are described elsewhere.[4] Samples were stored at −15° until dissolved and/or diluted for Raman study. All solvents employed were reagent grade. Protoporphyrin IX dimethyl ester and mesoporphyrin IX dimethyl ester were obtained from Sigma Chemicals, and deuteroporphyrin IX dimethyl ester was obtained from ICN-K&K Laboratories. Dissolving the cobalt porphyrin dimethyl esters in the solvents employed (piperidine, pyridine, *N,N*-dimethyl formamide (DMF), and acetonitrile) generally yielded solutions of the cobalt(II) oxidation state, while nonesterified cobalt porphyrins generally autoxidized to cobalt(III) upon dissolution. The esterification or lack thereof of the porphyrin side chains has no effect on the observed resonance Raman spectra. Solutions of cobalt(II) porphyrin dimethyl esters were oxidized, when necessary, using ceric perchlorate.

Raman spectra were taken using 5145 Å excitation in all cases, with the apparatus and scattering geometry described elsewhere.[38] Spectra were taken both digitally under on-line computer control,[39] and using conventional synchronous scan and analog recording techniques. Visible and ultraviolet spectra of Raman samples were obtained using a Cary 14 spectrophotometer. The total cobalt heme concentration for both the protein and metalloporphyrin solution spectra was $2\text{–}4 \times 10^{-4}$ *M*.

## Results

Figure 1 shows structural formulas of the three cobalt porphyrins examined, i.e., CoPP, CoDP, and CoMP. Figure 2 presents typical resonance Raman spectra of these metalloporphyrins, each as the heme group of the corresponding deoxy cobalt myoglobin. Most of the features in Figure 2 correlate in approximate frequency and in polarization among the three porphyrins. However, CoPP shows two bands which are not observed in CoMP viz. the depolarized mode at 1550 $cm^{-1}$ and the anomalously polarized mode at 1345 $cm^{-1}$. These extra bands have also been observed in comparing hemoglobin, in which the protoheme has two vinyl substituents, and cytochrome c, in which the protoheme vinyl groups are saturated by addition of the thioether links to the protein. For porphyrins in which all eight peripheral substituents are attached to the pyrrole rings by saturated carbon atoms, as in mesoporphyrin or the cytochrome c heme, the effective porphyrin symmetry is fourfold. Indeed we find that the resonance Raman spectrum of cobalt octaethylporphyrin, which has rigorous fourfold symmetry, is essentially the same as that of CoMP. The extra bands observed in protoporphyrin derivatives are attributable[38] to symmetry lowering by the two vinyl substituents, which are know to conjugate electronically with the $\pi$ system of the porphyrin. CoDP shows one extra band, the anomalously polarized band at 1343 $cm^{-1}$. In deuteroporphyrin, the vinyl groups are replaced by hydrogen, whose in-plane bending mode should couple effectively to vibrations of the ring, thereby providing a mechanism for symmetry lowering.

Table I compares the Raman frequencies observed for

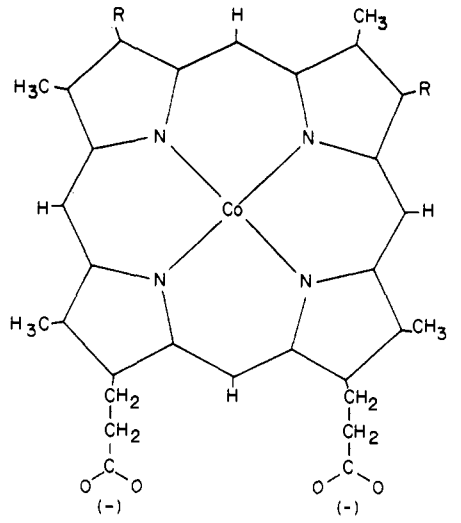


**Figure 1.** Structure of cobalt hemes, indicating the pyrrole substituents in protoporphyrin IX, deuteroporphyrin IX, and mesoporphyrin IX.

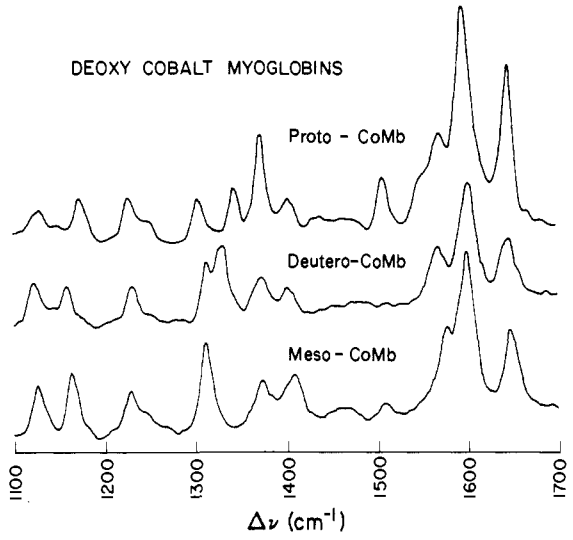


**Figure 2.** Resonance Raman spectra of deoxy cobalt myoglobins. The concentration of cobalt heme is $4 \times 10^{-4}$ *M* in each case. Frequencies are summarized and indicator bands are identified in Tables II and III. Spectra were obtained under on-line computer control, with points taken digitally at 1-$cm^{-1}$ intervals. At each frequency, 400 analog-to-digital conversions were averaged to suppress random noise. Instrumental conditions: 5145 Å excitation, 6-$cm^{-1}$ slit width, sensitivity $10^{-9}$ A full scale, excitation power approximately 50 mW.

$Co^{II}PP$ as the prosthetic group in deoxyproto-CoHb (reported previously[39]) and deoxyproto-CoMb, and also for solutions of $Co^{II}PP$ in the coordinating solvents piperidine, pyridine, DMF, and acetonitrile. There are few significant, systematic differences in the $Co^{II}PP$ frequencies observed among these six environments. The depolarized feature at approximately 1640 $cm^{-1}$ occurs at slightly higher frequency in solutions of the weaker bases DMF and acetonitrile than in piperidine and pyridine. This is the most conspicuous difference among the spectra in the four solvents, despite immense differences in the solvents' Bronsted basicity, $\pi$ bonding ability, and steric requirements.[21,24,29] The only consistent difference between the protein and solution spectra is in the position of the inversely polarized band at 1300–1310 $cm^{-1}$, which appears at approximately 6 $cm^{-1}$ higher frequency in the solutions. The proto-CoHb and proto-CoMb spectra are essentially the same, except for the depolarized vibration at 1558 $cm^{-1}$ in the former and 1550 $cm^{-1}$ in the latter.

Table I. Raman Frequencies (1100–1700 $cm^{-1}$) for Cobalt(II) Protoporphyrin IX in Various Media

| Deoxy-CoHb[a] | Deoxy-CoMb | Piperidine | Pyridine | DMF[b] | Acetonitrile | Polarization[c] |
|---|---|---|---|---|---|---|
| 1643 | 1645 | 1642 | 1641 | 1647 | 1646 | dp |
| 1593 | 1595 | 1593 | S | 1596 | 1594 | ap |
| 1570 | 1570 | 1568 | S | | | p |
| 1558 | 1550 | 1554 | S | | | dp |
| 1508 | 1508 | 1508 | 1506 | S | 1508 | p |
| 1401 | 1403 | 1401 | | S | S | ap |
| 1373 | 1373 | 1373 | 1374 | S | S | p |
| 1341 | 1343 | 1345 | | 1344 | S | ap |
| 1304 | 1303 | 1310 | | 1309 | 1309 | ap |
| 1244 | 1246 | S[d] | | | | dp |
| 1228 | 1228 | S | S | 1233 | | dp |
| 1172 | 1174 | 1170 | | 1174 | 1170 | dp |
| 1126 | 1126 | 1128 | | 1125 | | |

[a] Data from ref 39. [b] *N,N*-Dimethylformamide. [c] dp = depolarized, p = polarized, ap = anomalously polarized. [d] S = obscured by solvent band.

Because the Raman spectrum of CoPP is so similar in all of the solvents examined, there are no special constraints upon choice of solvent for comparisons between solution and protein spectra. Therefore piperidine was chosen, because its Raman spectrum interferes least with that of CoPP (see Table I). Piperidine forms both mono and bis complexes with cobalt(II) porphyrins, and at room temperature in piperidine solvent, the equilibrium lies about halfway between the five- and six-coordinate forms.[21] Higher temperatures favor the five-coordinate form, however. Our examination of the Soret band[21] of $Co^{II}MP$ in piperidine as a function of temperature indicates that, under the laser-heated conditions of the resonance Raman experiments, the metalloporphyrin is primarily (~90%) five coordinate.

Tables II and III compare the Raman frequencies of CoPP, CoDP, and CoMP between the piperidine solutions and the corresponding myoglobins. The previously reported proto-CoHb data[39] are also included. Table II compares the piperidine solutions of cobalt(II) porphyrins with the deoxy myoglobins and deoxyproto-CoHb. In Table III the cobalt(III) porphyrin solutions are compared with the oxy myoglobins and oxyproto-CoHb. In each table, the indicator bands are labeled to conform with previous notation.[38]

## Discussion

The frequencies which are monitored in this study arise from a series of in-plane porphyrin ring vibrational modes,[38] whose Raman intensities are enhanced by resonance with the dominant $\pi$-$\pi^*$ electronic transitions.[40,41] For CoPP these frequencies are quite insensitive to whether the solvent, which also acts as axial ligand, is piperidine, pyridine, DMF, or acetonitrile. Only the highest frequency, dp, mode varies by as much as 6 $cm^{-1}$. Evidently neither environmental effects nor specific axial ligand electronic effects influence these frequencies significantly, despite the widely differing properties of these solvents.

No significant changes are observed when CoPP is inserted in functioning hemoglobin or myoglobin. While alterations in the peripheral substituents, in the series proto-deutero- and mesoporphyrin, do affect the vibrational pattern, no significant changes are observed when the cobalt(II) derivative of any of the three is inserted in myoglobin. Nor are

Table II. Raman Frequencies (1300–1700 $cm^{-1}$) for Cobalt(II) Porphyrins in Hemoglobin, Myoglobin, and Piperidine

| Porphyrin | Medium: Deoxy hemoglobin[a] (label[b]) | Deoxy myoglobin | $Co^{II}P$ in piperidine | Polarization[c] |
|---|---|---|---|---|
| Proto | 1643 (F) | 1645 | 1642 | |
| Deutero | | 1644 | 1641 | dp |
| Meso | | 1646 | 1642 | |
| Proto | 1593 (C) | 1595 | 1593 | |
| Deutero | | 1597 | 1595 | ap |
| Meso | | 1597 | 1594 | |
| Proto | 1570 (D) | 1570 | 1568 | |
| Deutero | | 1568 | 1568 | p |
| Meso | | 1576 | 1574 | |
| Proto[d] | 1558 (B) | 1550 | 1554 | dp |
| Proto | 1508 (E) | 1508 | 1508 | |
| Deutero | | | | p |
| Meso | | 1509 | 1510 | |
| Proto | 1401 | 1403 | 1401 | |
| Deutero | | 1401 | | ap |
| Meso | | 1401 | 1406 | |
| Proto | 1373 (A) | 1373 | 1373 | |
| Deutero | | 1372 | 1372 | p |
| Meso | | 1373 | 1374 | |
| Proto[e] | 1341 | 1343 | 1345 | |
| Deutero[e] | | 1330 | 1328 | ap |
| Proto | 1304 | 1303 | 1310 | |
| Deutero | | 1312 | 1312 | ap |
| Meso | | 1312 | 1312 | |

[a] Data from ref 39. [b] Reference 38. [c] dp = depolarized, p = polarized, ap = anomalously polarized. [d] Vibration appears only in protoporphyrin IX species (see text). [e] Vibration absent in mesoporphyrin IX species (see text).

Table III. Raman Frequencies (1300–1700 $cm^{-1}$) for Oxygenated Cobalt Hemes or Cobalt(III) Porphyrins in Hemoglobin, Myoglobin, and Piperidine

| Porphyrin | Medium: Oxy hemoglobin[a] (label[b]) | Oxy myoglobin | $Co^{III}P$ in piperidine | Polarization[c] |
|---|---|---|---|---|
| Proto | 1649 (F) | 1652 | 1645 | |
| Deutero | | 1651 | 1644 | dp |
| Meso | | 1652 | 1644 | |
| Proto | 1596 (C) | 1600 | 1596 | |
| Deutero | | 1603 | 1600 | ap |
| Meso | | 1600 | 1592 | |
| Proto | 1572 (D) | 1572 | | |
| Deutero | | 1574 | 1572 | p |
| Meso | | 1580 | 1577 | |
| Proto[d] | 1560 (B) | 1560 | 1554 | dp |
| Proto | 1512 (E) | 1512 | 1509 | |
| Deutero | | | 1510 | p |
| Meso | | 1508 | 1520 | |
| Proto | 1402 | 1402 | | |
| Deutero | | 1407 | 1404 | ap |
| Meso | | 1412 | 1414 | |
| Proto | 1380 (A) | 1380 | 1379 | |
| Deutero | | 1387 | 1383 | p |
| Meso | | 1384 | 1382 | |
| Proto[e] | 1344 | 1348 | 1348 | ap |
| Deutero[e] | | 1332 | 1335 | |
| Proto | 1304 | 1312 | 1310 | |
| Deutero | | 1313 | 1316 | ap |
| Meso | | 1315 | | |

[a] Data from ref 39. [b] Reference 38. [c] dp = depolarized, p = polarized, ap = anomalously polarized. [d] Vibration appears only in protoporphyrin IX species (see text). [e] Vibration absent in mesoporphyrin IX species (see text).

large differences observed when the cobalt(III) derivatives are compared with the corresponding oxy-cobalt myoglobins.

Crystal structure data show that the distance between the mean porphyrin plane and the nitrogen atom of an axial imidazole ligand in five-coordinate cobalt(II) porphyrins is 2.30–2.40 Å.[17,18] The corresponding distance in six-coordinate cobalt(III) porphyrins is 1.93 Å.[15,17] This difference of 0.37–0.47 Å has been taken by Ibers and coworkers[15,18,23] to represent the maximum displacement of the proximal histidine residue with respect to the mean heme plane upon oxygenation of CoHb. This is sufficiently smaller than the corresponding displacement in FeHb, estimated to be 0.8–0.9 Å, to raise a question about the movement of the proximal histidine being the stereochemical trigger for cooperativity. Hoard and Scheidt,[14] on the other hand, have proposed that constraints imposed by the protein could increase the histidine displacement in CoHb to about 0.8 Å. This would be accomplished mainly by doming of the porphyrin ring in deoxy CoHb by an amount as great as, or greater than, that of FeHb.

This degree of doming should be clearly evident in the resonance Raman spectra, however. In iron porphyrins large frequency shifts, up to 30 $cm^{-1}$, are observed for certain of the Raman bands, upon changing spin state.[38] These shifts are almost certainly a consequence of the altered stereochemistry of the porphyrin ring, which is planar for low-spin and domed for high-spin iron derivatives.[42,43] Purely electronic effects of a change in spin state should be less than those of a change in oxidation state, but the frequency shifts accompanying the latter are relatively small.[38] The view that the spin-state indicator bands reflect porphyrin stereochemistry primarily gains support from a recent report[44] that the position of the most characteristic of these bands, the ~1590 $cm^{-1}$ ap band (C), correlates with out-of-plane displacement for other metal ions ($VO^{2+}$ and $Zn^{2+}$), which are not involved in spin-state changes.

It was initially inferred on the basis of similar electron spin resonance spectra[2a,26] that CoPP in hemoglobin is structurally similar to five-coordinate CoPP in solution. The esr spectrum monitors the unpaired electron distribution, which is centered primarily on the cobalt atom. It gives no direct information about porphyrin conformation, although changes in the latter would very likely affect the esr parameters. Resonance Raman spectroscopy monitors porphyrin ring vibrational frequencies, which should be directly a function of porphyrin conformation. In our previous study[39] we argued on the basis of frequency *shifts* accompanying oxygenation of CoHb, compared to the corresponding shifts in FeHb, that no significant porphyrin doming occurs in CoHb. Fragmentary data for cobalt porphyrins were included for comparison. In the present study we have carefully examined all of the resonance enhanced porphyrin modes of CoPP in various solutions and in both myoglobin and hemoglobin. The near identity of *all* vibrational frequencies in the protein matrices and in solution establishes, quite conclusively, that neither myoglobin nor hemoglobin induces an appreciable distortion in the porphyrin ring of CoPP. The same conclusion applies with respect to myoglobin and CoDP or CoMP.

Earlier we suggested,[39] on the basis of Hopfield's linear distributed energy model,[45] that the 0.37–0.47 Å histidine to mean heme plane displacement, estimated by Ibers and coworkers for CoHb, is not inconsistent with Perutz' stereochemical trigger hypothesis for cooperative oxygen binding. According to Hopfield's model, which incorporates the stereochemical trigger, the free energy of cooperativity should be proportional to the histidine to mean heme plane displacement. For CoHb the free energy of cooperativity appears to be only about one-third that of FeHb.[39] Since 0.37–0.47 Å is more than one-third the estimated displace-

ment in FeHb, the model is satisfied without any distortion in the porphyrin ring.

On the other hand Little and Ibers[18] have pointed out that if the histidine to mean heme plane displacement were the sole determinant of the quaternary structure transition which produces cooperativity, then deoxy-CoHb should have the same quaternary structure as aquomet-FeHb, inasmuch as the distance from the histidine nitrogen to the mean porphyrin plane is ~2.3 Å in both. Yet aquomet-Fehb is in the oxy quaternary structure (although it can be switched to the deoxy quaternary structure by binding of organic phosphates) while deoxy-CoHb is firmly in the deoxy quaternary structure. Little and Ibers suggest that interaction with the distal histidine via the water molecule bound as a sixth ligand to met-FeHb may account for the difference, since deoxy-CoHb is probably five coordinate. Another caveat emerges from the work of Yonetani *et al.*[4] on cooperativity in hemoglobins reconstituted with porphyrins containing different peripheral substituents. For both iron and cobalt hemoglobins, cooperativity varied in the order proto-Hb > deutero-Hb > meso-Hb. The present study shows no structure change for any of the three cobalt porphyrins on binding to myoglobin. The same conclusion no doubt holds for hemoglobin, although data are available only for cobalt proto-Hb. It therefore appears, at least for CoHb, that the observed cooperativity variations must arise from direct interactions of the peripheral substituents with the protein, rather than from indirect effects on porphyrin structure. Evidently factors other than histidine to mean heme plane displacement must be included in a complete account of hemoglobin cooperativity.

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