Modeling Low-pH Hemoproteins*

(Received for publication, June 29, 1989)

Laura A. Andersson† and Muthusamy Mylrajans§
From the Department of Chemical and Biological Sciences, Oregon Graduate Center, Beaverton, Oregon 97006-1999

Eric P. Sullivan, Jr. and Steven H. Strauss
From the Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

A tetracoordinate ferrous heme (iron-porphyrin) has been proposed as an intermediate at low pH (<3.0) for respiratory hemoproteins, peroxidases, and model heme complexes. This intermediate is believed to arise via protonation of the N(ε) atom of the proximal histidine and consequent cleavage of the Fe–N(ε) bond. To establish a spectral signature for the proposed low-pH tetracoordinate species, we have obtained Soret excitation resonance Raman spectra on samples of crystallographically defined, tetracoordinate iron(II)-octaethylporphyrin (Fe-OEP; S = 1). The high-frequency (≥900 cm⁻¹) resonance Raman spectral features of Fe-OEP are clearly distinct from those of high-spin pentacoordinate or low-spin hexacoordinate ferrous hemes. Rather, they are at frequencies more typically observed for low-spin hexacoordinate ferric porphyrins. Comparative spectral analysis of tetracoordinate Fe-OEP and other proposed tetracoordinate ferrous hemes (e.g., iron(II)-protoporphyrin IX) demonstrates little or no macrocycle effect on the resonance Raman frequencies above 900 cm⁻¹. This work thus serves to provide a testable spectral signature by which the existence of the proposed tetracoordinate biological intermediate may be verified and by which its functional significance may be tested.

Kinetic studies of myoglobin and other monomeric respiratory hemoproteins (e.g., Refs. 1-6), hemoglobin (7), peroxidases (8, 9), and model heme (iron-porphyrin) compounds (10-13) have led to suggestions of a tetracoordinate ferrous iron intermediate at low pH (<3.0). For the proteins, this species is presumed to be derived via protonation of the proximal imidazole ligand to the ferrous heme, resulting in cleavage of the Fe–N(ε) bond, as was first shown to be the case for the model complexes (10-13).

The respiratory hemoproteins and the model complexes demonstrate a remarkably altered affinity for CO at low pH, for which the proposed tetracoordinate intermediate has offered an attractive and apparently plausible explanation. Loss of the bond between the proximal imidazole and the ferrous iron of the hemoproteins has been suggested to lead to a conformational transition, resulting in the observed rate increase for CO binding at low pH (6), as was first reported for the model complexes (10-13). In the case of the peroxidases, cleavage of the proximal Fe–N(ε) bond is also proposed (8, 9); however, no increase in CO binding affinity was observed (9).

Of particular interest, the spectral changes occurring in hemoglobin upon lowering the pH parallel those observed upon addition of allosteric effectors such as inositol hexaphosphate. This has led to proposals that the structure at the proximal site might correlate with quaternary conformational changes (14-16). Indeed, the ~5 kcal/mol energy involved in the Fe–N(ε) bond (3) is of the same order of magnitude as the T-to-R quaternary transition of hemoglobin (17). Thus, it has been suggested that cleavage of the Fe–N(ε) bond might possibly occur in the T quaternary conformation for hemoglobin and thereby explain the different CO binding constant between the R and T forms of the respiratory heme protein (1). The hypothesis of a tetracoordinate intermediate is proposed to involve a more planar heme conformation, thereby enhancing the CO reactivity independently of a quaternary modulation (1).

Resonance Raman spectroscopy is well known to be a useful probe of biological and model heme systems (18-20). This is particularly true with respect to identification of oxidation state (reflecting probe electron density at the central iron), spin state, and coordination number for heme enzymes (18-23). Furthermore, resonance Raman spectroscopy has been valuable in the study of metal–ligand bonds, especially the Fe–N(histidine) mode of respiratory hemoproteins (20, 24, 25) and of enzymes such as cytochrome-c peroxidase (26). Consequently, resonance Raman spectroscopy offers an ideal means for testing the existence of the proposed low-pH tetracoordinate ferrous heme intermediate of model and biological systems if a suitable spectral signature is established.

To that end, we have obtained the Soret excitation resonance Raman spectra of samples that have been structurally defined as intermediate-spin (S = 1) tetracoordinate ferrous heme: iron(II)-octaethylporphyrin (Fe-OEP)¹ (27). The resonance Raman spectral frequencies of tetracoordinate Fe-OEP are clearly distinct from the well-known bands of high-spin pentacoordinate and low-spin hexacoordinate ferrous hemes. Accordingly, the spectral data presented herein should aid in analysis of the proposed tetracoordinate ferrous heme intermediate generated at low pH from ferrous hemoproteins and model complexes.

¹ The abbreviations used are: OEP, octaethylporphyrin; MP, mesoporphyrin IX; PP, protoporphyrin IX; TPP, meso-tetraphenylporphyrin.
EXPERIMENTAL PROCEDURES

Resonance Raman spectra were obtained from powder samples of Fe-OEP, prepared as previously described (27) and sealed under N₂ in capillary tubes. Samples of Fe-OEP in benzene or dichloromethane (−1 mol) were prepared anaerobically and sealed in NMR tubes under N₂, were also examined. All manipulations and physical measurements were performed with rigorous exclusion of dioxygen and water. The samples were maintained at 280 K in a Dewar flask with a copper cold finger (28), resonance Raman data were collected using backscattering geometry. Soret (413.1 nm) excitation was provided by a Spectra-Physics 2025-11 krypton ion laser. The Raman spectroscopic and computer interface have been described previously (29) and were recently upgraded (30).

RESULTS AND DISCUSSION

The oxidation state, spin state, and coordination number of both model and biological iron-porphyrin systems display characteristic and generally diagnostic resonance Raman frequencies (18–20). For example, the v₂ marker band, a particularly useful feature, appears at ~1470–1475 cm⁻¹ for high-spin pentacoordinate ferrous hemes, at ~1490 cm⁻¹ for low-spin hexacoordinate ferrous hemes, and at ~1500 cm⁻¹ for low-spin hexacoordinate ferric hemes (see also Table I). It is precisely the generality of these resonance Raman features that has rendered them so useful in the study of biological heme systems (23).

The first structurally characterized four-coordinate iron (II)-porphyrin was the meso-tetraphenylporphyrin complex, Fe-TPP (31). The iron atom was shown to be precisely in-plane, with an Fe(II)–N₄porphyrin bond distance of 1.972 (4) Å (31). Burke et al. (32) have reported the resonance Raman spectrum of intermediate-spin Fe-TPP generated in situ by reduction of a dichloromethane solution of the iron(III) complex. The resonance Raman spectral frequencies of Fe-TPP were very similar to those of low-spin iron(III)-TPP complexes (23). However, the resonance Raman spectral pattern of meso-substituted porphyrins is known, to be very different from that of "physiological" (β-pyrrole-substituted) porphyrins and heme proteins (32–35). This is primarily because differences in the placement of the peripheral substituents between the two types of porphyrins have a strong effect on the vibrational interactions between the macrocycle and its substituents (32), particularly in the case of meso-aryl substituents.

The Soret excitation resonance Raman spectrum of solid-state, crystallographically defined Fe-OEP (27) is shown in Fig. 1. The Soret (inset) presents the 150–375 cm⁻¹ resonance Raman spectrum of tetracoordinate Fe-OEP in benzene. Key resonance Raman frequencies and their assignments are listed in Table 1. This is the first report of the resonance Raman spectral properties of β-pyrrole-substituted iron(II)-porphyrin samples that are unequivocally known to be tetracoordinate ferrous hemes.

Solution resonance Raman spectra of heme complexes, proposed to be tetracoordinate ferrous species generated in situ, were reported previously (36–39). However, the challenges of maintaining a tetracoordinate iron(II)-porphyrin in solution are well known. Difficulties include the well-known proclivity of ferrous hemes to be oxidized or to bind ligands, requiring the rigorous exclusion of dioxygen and water. Furthermore, the commonly used solvent dichloromethane, which is now known to coordinate to metal ions (41), is also known to oxidize Fe-OEP (27). Thus, our resonance Raman spectral study of the structurally defined tetracoordinate iron(II)-porphyrin provides the benchmark by which the previous solution spectra of in situ generated iron(II)-porphyrins may be evaluated.

The high-frequency resonance Raman features of tetracoordinate Fe-OEP are clearly distinct from those of high-spin hexacoordinate ferrous heme systems (Table I). Therefore, one should readily be able to identify the presence of an in situ generated tetracoordinate iron(II)-porphyrin at low pH and distinguish it from the pentacoordinate ferrous heme system from which it is presumably derived. The high-frequency resonance Raman features of tetracoordinate Fe-OEP also differ from those of low-spin hexacoordinate ferrous heme systems, such as ferrocychrome b₅ (Table I) or ferrocytochrome c (18).

The resonance Raman features of Fe-OEP generated in situ in dichloromethane (36) differ from those of solid-state Fe-OEP by 2–4 cm⁻¹ (Table I). However, 2–5 cm⁻¹ differences are also observed between solid-state Fe-OEP and its benzene solution sample (Table I). Thus, despite the fact that dichloromethane is not an "innocent solvent" (27, 41), the observed frequency variations do not provide evidence for solvent-induced alterations in the oxidation state or spin state of the ferrous porphyrins. Similarities among the resonance Raman frequencies of Fe-OEP, iron(II)-mesoporphyrin IX (Fe-MP), and iron(II)-protoporphyrin IX (Fe-PP) thus indicate that in situ generated iron(II)-porphyrins previously reported were predominantly tetracoordinate species as proposed (36–39), regardless of the presence of dichloromethane and its attendant difficulties (27, 41).

Comparison of the resonance Raman frequencies of the structurally defined tetracoordinate Fe-OEP complex with those of the tetracoordinate ferrous forms of physiologically relevant heme model complexes, Fe-MP and Fe-PP, reveals considerable similarity (Table I). These data indicate that the nature of peripheral substituents, between β-pyrrole-substituted heme complexes, has no significant effect on resonance Raman frequencies of ≥900 cm⁻¹ for the four-coordinate ferrous state. Consequently, the data reported herein for Fe-OEP should be relevant in the examination of proposed low-spin intermediates of biological systems.

The high-frequency resonance Raman bands of tetracoordinate Fe-OEP are perhaps most similar to those of low-spin hexacoordinate ferric hemes (Table I). Correspondences between the resonance Raman frequencies of a low-spin hexacoordinate ferric heme and the tetracoordinate complex of Fe-MP in dichloromethane solution (Table I) were first noted by Spiro and Burke (37). This observation was suggested to be consistent with the S = 1 spin state of the tetracoordinate ferrous heme (37). Kitagawa and co-workers subsequently reported solution spectra for tetracoordinate Fe-OEP (36) and for Fe-PP (38). Kitagawa and Terasaki (36) suggested that the 4E_v ((d_0^2) (d^2) d_0^2) state was the most compatible intermediate-state state for tetracoordinate iron(II)-porphyrins. This state for an intermediate-spin ferrous porphyrin is expected to have an empty d^2 orbital, as previously noted (37). The electronic configuration of the intermediate-spin tetracoordinate ferrous heme is thus similar to that of a low-spin hexacoordinate ferric heme, which also has an empty d^2 orbital.

Calculations for intermediate-spin iron(II)-porphyrine by Ed-

* A detailed analysis of the spectral properties of Fe-OEP, along with those of iron(I)-octaethylchlorin and iron(II)-octaethylisobacteriochlorin, is in progress and will address core size/resonance frequency correlations, solution versus solid-state effects, and temperature effects (M. Mylrijan, L. A. Andersson, T. M. Loehr, E. P. Sullivan, Jr., and S. H. Strauss, manuscript in preparation).

2 The v₂ vibrational mode is observed at ~1640 cm⁻¹ for low-spin hexacoordinate ferrous systems where the sixth ligand is a σ-acid, e.g. O₂, NO, or CO (18–20). Similarly, the frequency of v₄ is at ~1370 cm⁻¹ for these systems.
Fig. 1. Soret excitation resonance Raman spectrum of solid-state, tetracoordinate Fe*OEP. Inset, Fe*OEP at ~1 mM in benzene solution. Conditions: laser power, 20 milliwatts at sample; backscattering geometry; and scan rate, 1 cm⁻¹/s.

### Table I

<table>
<thead>
<tr>
<th>Selected resonance Raman frequencies of heme systems</th>
<th>( \nu_1 ) (dp)</th>
<th>( \nu_2 ) (dp)</th>
<th>( \nu_3 ) (dp)</th>
<th>( \nu_0 ) (dp)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tetracoordinate ferrous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe*OEP (solid-state)</td>
<td>1375</td>
<td>1500</td>
<td>1590</td>
<td>1637</td>
<td>This work</td>
</tr>
<tr>
<td>Fe*OEP (benzene solution)</td>
<td>1377</td>
<td>1505</td>
<td>1591</td>
<td>1639</td>
<td>This work</td>
</tr>
<tr>
<td>Fe*OEP (CH₂Cl₂ solution)</td>
<td>1377</td>
<td>1504</td>
<td></td>
<td>1640</td>
<td>36</td>
</tr>
<tr>
<td>Fe*MP (CH₂Cl₂ solution)</td>
<td>1373</td>
<td>1506</td>
<td>1596</td>
<td>1642</td>
<td>37</td>
</tr>
<tr>
<td>Fe<strong>PP (2.5% CTAB-solubilized solution)</strong></td>
<td>1372</td>
<td>1500</td>
<td></td>
<td>1638</td>
<td>38</td>
</tr>
<tr>
<td>Fe<strong>PP (SDS-solubilized)</strong></td>
<td>1368</td>
<td>1501</td>
<td>1586</td>
<td>1640</td>
<td>39</td>
</tr>
<tr>
<td><strong>Pentacoordinate ferrous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deoxy-Mb</td>
<td>1357</td>
<td>1473</td>
<td>1563</td>
<td>1607</td>
<td>40</td>
</tr>
<tr>
<td>Deoxy-Hb</td>
<td>1358</td>
<td>1473</td>
<td></td>
<td>1607</td>
<td>18</td>
</tr>
<tr>
<td>Fe<strong>PP (2-Melm)</strong></td>
<td>1357</td>
<td>1471</td>
<td>1562</td>
<td>1604</td>
<td>40</td>
</tr>
<tr>
<td>Fe*OEP (THF)</td>
<td>1364</td>
<td></td>
<td></td>
<td>1610</td>
<td>36</td>
</tr>
<tr>
<td><strong>Hexacoordinate ferrous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome b₅</td>
<td>1361</td>
<td>1493</td>
<td></td>
<td>1617</td>
<td>18</td>
</tr>
<tr>
<td>Fe**PP ((Im)₅)</td>
<td>1359</td>
<td>1495</td>
<td>1570</td>
<td>1620</td>
<td>40</td>
</tr>
<tr>
<td><strong>Pentacoordinate ferric</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe*PP (Cl⁻)</td>
<td>1373</td>
<td>1491</td>
<td>1570</td>
<td>1626</td>
<td>40</td>
</tr>
<tr>
<td>A. niger catalase</td>
<td>1373</td>
<td>1489</td>
<td>1574</td>
<td>1625</td>
<td>23</td>
</tr>
<tr>
<td><strong>Hexacoordinate ferric</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MetMb (CN⁻)</td>
<td>1374</td>
<td>1506</td>
<td>1583</td>
<td>1642</td>
<td>21</td>
</tr>
<tr>
<td>LiP (CN⁻)</td>
<td>1373</td>
<td>1500</td>
<td>1584</td>
<td>1639</td>
<td>21</td>
</tr>
<tr>
<td>Fe**PP ((Im)₆)</td>
<td>1373</td>
<td>1502</td>
<td>1579</td>
<td>1640</td>
<td>40</td>
</tr>
</tbody>
</table>

*p*, polarized; dp, depolarized; CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate; Mb, myoglobin; Hb, hemoglobin; 2-Melm, 2-methylimidazole; THF, tetrahydrofuran; Im, imidazole; A. niger catalase, Aspergillus niger catalase; LiP, lignin peroxidase.

wards et al. (42) indicate that the \( \tilde{A}_2 \) \((d_{xy})^2(d_{yz})^2(d_{zx})^2\) state is 240 cm⁻¹ below the \( E_g \) state. Nonetheless, based on analysis of x-ray and Mössbauer data, they proposed a \( E_g \) ground state (42). In contrast, the \( \tilde{A}_2 \) state was favored by Goff et al. (43). In a recent analysis of \( S = 1 \) tetracoordinate ferrous porphyrins, McGarvey (44) proposed a \( \tilde{A}_2 \) ground state that is spin orbit-coupled with the \( E_g \) ground state. NMR and magnetic susceptibility results are consistent with a ground state that includes both \( \tilde{A}_2 \) and \( E_g \) character (27, 45).

In the low-frequency (≤900 cm⁻¹) resonance Raman spectrum of Fe*OEP, bands at 344, 668, and 805 cm⁻¹ for the solid-state Fe*OEP sample may be assigned as \( v_7 \), \( v_7 \), and \( v_6 \), respectively. Because the solid-state, anaerobically sealed Fe*OEP complex is known to be tetracoordinate, the weak feature at 212 cm⁻¹ cannot be a metal-ligand vibrational mode. This band is present at 209 cm⁻¹ for the benzene solution sample of Fe*OEP, as shown in Fig. 1 (inset), and presumably correlates with the ~202 cm⁻¹ band of Ni*OEP, assigned as \( v_{14} \). However, the low-frequency solution spectrum of Fe*PP does not display a band in this region (38). The solution sample of Fe*OEP has a feature at 254 cm⁻¹, assigned as \( v_6 \) for Ni*OEP, that is considerably weaker for the solid-state Fe*OEP sample (Fig. 1). The intensity change for this band is the major difference between the low-frequency spectra of the solid-state and solution samples of Fe*OEP. Bands in this region have also been assigned as in-plane skeletal modes (46) and out-of-plane skeletal modes, and some M–N(pyrrole) stretching contribution may also be present (46).

### CONCLUSIONS

The high-frequency resonance Raman spectral properties of samples of structurally defined intermediate-spin iron(II)-octaethylporphyrin are shown to be clearly distinct from those of high-spin pentacoordinate iron(II)-heme proteins and model complexes. They also differ from those of low-spin hexacoordinate iron(II)-heme systems. Instead, the resonance Raman frequencies of tetracoordinate Fe*OEP are most similar to the features of low-spin hexacoordinate ferric porphyrins. The data presented herein for solid-state tetracoordinate Fe*OEP are comparable to those reported previously for in situ generated solution samples of \( \beta \)-pyrrole-substituted iron(II)-porphyrins, regardless of the identity of the peripheral substituents. We have established a resonance Raman spectral signature for tetracoordinate iron(II)-porphyrins that...
should be invaluable in the study of species induced at low pH for respiratory hemoproteins, peroxidases, and their model complexes. This work should thus aid in the elucidation of the functional significance of the proposed biological tetra-coordinate ferrous heme intermediate.

Acknowledgments—We thank Professor Thomas M. Loehr and Dr. Denia L. Rousseau for stimulating and insightful discussions.

REFERENCES