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Using Raman Spectroscopy To Monitor the Solvent-Exposed and "Buried" Forms of Flavin in *p*-Hydroxybenzoate Hydroxylase†

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ABSTRACT: X-ray crystallographic studies of several complexes involving FAD bound to *p*-hydroxybenzoate hydroxylase (PHBH) have revealed that the isoalloxazine ring system of FAD is capable of adopting in two positions on the protein. In one, the "in" form, the ring is surrounded by protein groups and has little contact with solvent; in the second, "out" form, the ring is largely solvent exposed. Using Raman difference spectroscopy, it has been possible to obtain Raman spectra for the flavin ring in both conformational states for different complexes in solution. The spectra consist of a rich assortment of isoalloxazine ring modes whose normal mode origin can be assigned by using density functional theory and ab initio calculations. Further insight into the sensitivity of these modes to changes in environment is provided by the Raman spectra of lumiflavin in the solid state, in DMSO and in aqueous solution. For the protein complexes, the Raman difference spectra of flavin bound to wt PHBH and wt PHBH plus substrate, *p*-hydroxybenzoate, provided examples of the "in" conformation. These data are compared to those for flavin bound to wt PHBH plus 2,4-dihydroxybenzoate, where X-ray analysis show that the flavin is "out". There are several spectral regions where characteristic differences exist for flavin in the "in" or "out" conformation, these occur near 1700, 1500, 1410, 1350, 1235, and 1145 cm^{-1}. These spectral features can be used as empirical marker bands to determine the populations of "in" and "out" for any complex of PHBH and to monitor changes in those populations with perturbations to the system, e.g., by changing temperature or pH. Thus, it will now be possible to determine the conformational state of the flavin in PHBH for those complexes that have resisted X-ray crystallographic analysis. Raman difference data are also presented for the Tyr222Phe mutant. The Raman data show that the isoalloxazine ring is predominantly "out" for Tyr222Phe. However, in the presence of the substrate *p*-hydroxybenzoate there is clear evidence from the Raman marker bands that a mixed population of "in" and "out" exists with the majority being in the "out" state. This is consistent with the conclusions drawn from crystallographic studies on this complex (Gatti, D. L., Palfey, B. A., Lah, M. S., Entsch, B., Massey, V., Ballou, D. P., and Ludwig, M. L. (1994) *Science*, *266*, 110−114).

p-Hydroxybenzoate hydroxylase (EC 1.14.13.2) catalyzes the reaction of *p*-hydroxybenzoate, O_2, and NADPH to form 3,4-dihydroxybenzoate, water, and $NADP^+$ (*1*−*2*). In the absence of the cofactor NADPH, the enzyme forms a

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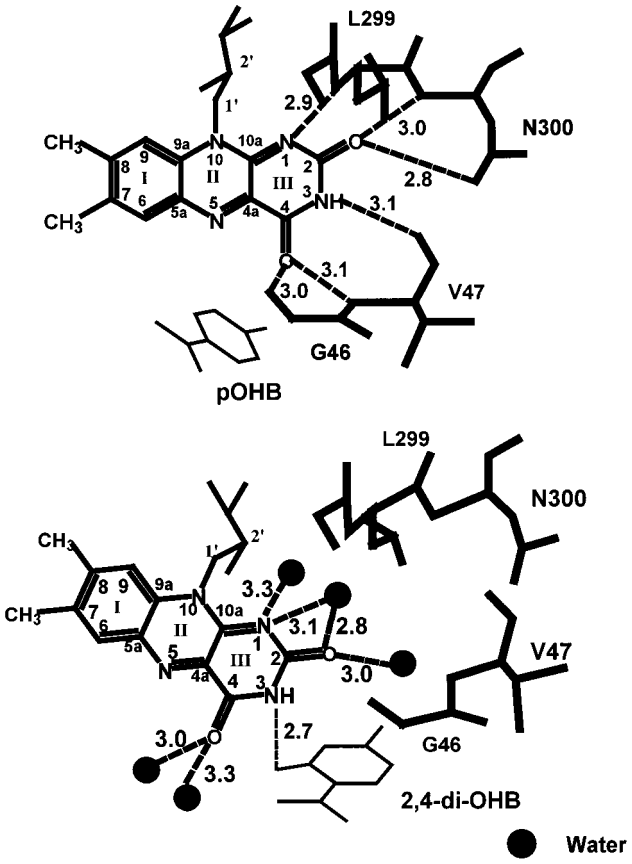

FIGURE 1: Structures of the active sites of the PHBH complexes, showing the flavin "in" (top portion) conformation and "out" (lower portion) conformation. Structures were obtained from the Protein Data Bank. 1PBE: WT-PHBH−pOHB complex. 1DOD: WT-PHBH−2,4-di-OHB complex.

nonreacting 1:1 complex with its substrate. A large number of crystal structures of PHBH have been determined, with a variety of aromatic ligands bound to the wild-type or mutant enzymes (*3*−*8*). In the majority of structures, the reactive isoalloxazine moiety of the flavin is largely buried and is adjacent to the aromatic substrate in a position ideal for the hydroxylation reaction. Only the dimethyl edge and some of the *re* face are exposed to the solvent. However, a number of structures exhibit an alternate flavin conformation in which the isoalloxazine rotates by ~33° about the $C_{1'}-C_{2'}$ ribityl bond into a largely solvent-exposed position. These two conformations are referred to as "in" and "out", respectively (see Figure 1). The existence of two conformations might provide a means of explaining how PHBH stabilizes the diversity of transition states required in its catalytic cycle. While geometric constraints clearly require that the hydroxylation reaction occurs with the flavin in the "in" conformation, it has been proposed that flavin reduction by NADPH occurs in the "out" conformation (*9*, *10*). "In" and "out" conformations similar to those seen in PHBH have also been observed in phenol hydroxylase (*11*), suggesting that flavin conformational mobility is a general property of the enzyme family.

Using 752 nm excitation and a recently developed high throughput Raman spectrometer (*12*−*14*), very high quality spectra of the bound flavin can be generated by Raman difference spectroscopy. This experiment takes the form of subtracting the spectrum of the apoenzyme from that with the flavin bound. The Raman spectrum of a flavin is dominated by the isoalloxazine modes and is sensitive to changes in the ring microenvironment in the active site. We are able to detect clear differences between the flavin environments of the "in" and "out" conformations by Raman spectroscopy. Interpretation of the isoalloxazine spectra, combined with detailed quantum mechanical calculations, may in future provide a route to understanding how the protein controls flavin reactivity in each environment.

MATERIALS AND METHODS

p-Hydroxybenzoic acid and 2,4-di-OH-benzoic acid were from Aldrich. Lumiflavin and FAD were purchased from Sigma. All were used without further purification.

Preparation of Enzyme Samples. PHBH cloned from *Pseudomonas auerugenosa* was expressed in *Escherichia coli* (*15*) and purified as described previously (*16*). The plasmid for the Tyr222Phe form of PHBH is described in Gatti et al. (*6*). Apo-PHBH was prepared according to the protocol of Muller and van Berkel (*17*). To stabilize the apoenzyme, pOHB is included in all steps of its preparation. pOHB was removed by precipitating the apoenzyme with 70% saturated ammonium sulfate, centrifuging, and resuspending the apoenzyme in fresh pOHB-free 70% saturated ammonium sulfate; this process was repeated at least four times, and the apoenzyme was stored as a precipitate in the presence of 70% saturated ammonium sulfate. For spectroscopy, apoenzyme was centrifuged, the pellet was dissolved in 100 mM potassium phosphate buffer, pH 7.5, and the solution was quickly filtered (0.22 μm) immediately before Raman spectra were collected.

Raman Spectroscopy. For the enzyme complexes, Raman spectra were acquired using 850 mW 752 nm laser excitation from an Innova 400 krypton laser (Coherent, Inc.) and a modified Holospec f/1.4 axial transmission spectrometer (*12*). Typically, sample volumes were approximately 50 μL, and the spectrum was recorded at 22 °C with the total data collection time of 10 min. The spectrum of the apoenzyme plus a stoichiometric amount of FAD was recorded immediately after recording the spectrum of the apoenzyme. The subtraction of [apoenzyme + FAD] minus [apoenzyme] gave the spectrum of bound FAD. The computer subtraction was undertaken using Grams/32 software (Galactic Industries Company). For some experiments the apoenzyme spectrum was recorded in the presence of a stoichiometric amount of ligand, e.g., *p*-OH-benzoic acid.

The Raman spectra of lumiflavin in DMSO or aqueous solution were recorded by subtracting the Raman spectrum of solvent from that of lumiflavin in the solvent. The Raman spectrum of lumiflavin as a powder was obtained using a Holoprobe optic fiber (Kaiser Optical System) with 785 nm diode laser excitation and 2 h data accumulation.

Vibrational Analysis. Harmonic frequency calculations of lumiflavin were carried out with the Gaussian 94 package (*18*) on a T90 vector processor (Ohio Supercomputer Center, Columbus) or a Silicon Graphics workstation in this laboratory. The geometry of each molecule was completely optimized at the appropriate level of theory by analytic

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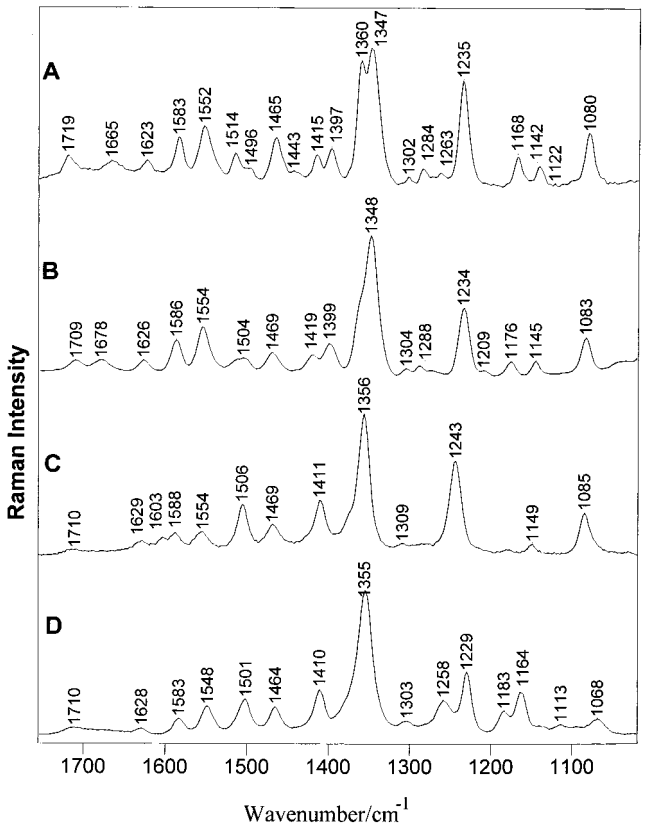

FIGURE 2: The Raman spectra of lumiflavin and FAD. (A) Lumiflavin in powder state. (B) Saturated solution of lumiflavin in d_6-DMSO, 850 mW 752 nm laser, 5 min binning. (C) Saturated solution of lumiflavin in 100 mM phosphate buffer, pH 7.5, 850 mW 752 nm laser, 20 min binning. (D) 300 μM FAD in 100 mM phosphate buffer, pH 7.5, 850 mW 752 nm laser, 10 min binning.

gradient techniques. A density functional theory (DFT) variant B3LYP with a basis set of 6-31G** was first employed to give normal modes. Then, a Hartree−Fock (HF) method with the same basis set was performed, producing Raman activities and nearly the same normal modes as those from DFT level. The calculated frequencies at the DFT/B3LYP level were converted by a single scaling factor (0.9614), according to a recent comprehensive evaluation of Scott and Radom (*19*).

RESULTS AND DISCUSSION

Development of Raman Marker Bands as Probes of the Flavin Environment. The Raman spectra of lumiflavin (N_{10}-methyl isoalloxazine) and FAD (the form bound to PHBH), generated by 752 nm excitation, are shown in Figure 2. The effect of changes in dielectric constant on the Raman spectra of lumiflavin was explored by recording data for the solid and for lumiflavin in DMSO and aqueous solution. Several Raman features were found to be sensitive to changes in solvent. These include bands near 1700 cm^{-1}, in the carbonyl region; the relative intensity of the peak near 1500 cm^{-1}; the presence of one or two bands between 1400 and 1420 cm^{-1}; the complex profile near 1350 cm^{-1}; the band near 1235 cm^{-1} and that near 1145 cm^{-1}.

Several earlier studies using isotopic substitution and semiempirical calculations provide assignment of flavin bands (*20*−*22*). We are now able to take advantage of improvements in theory and computational power to improve the confidence level in the assignments. Our approach depends on the use of density functional theory (Becke's three-parameter exchange functional in combination with the Lee−Yang−Parr correlation functional, using a basis set of 6-31G**, abbreviated as B3LYP/6-31G**) with the calculations being undertaken on a supercomputer. The calculations are able to reproduce the experimental results of other workers who observed the effect of placing ^2H, ^{13}C, ^{18}O, and ^{15}N at various positions in the ring (*20*). The assignments for the Raman bands in the 1000−1800 cm^{-1} region are given in Table 1. The numbering scheme for the flavin ring is shown in Figure 1. Most of our assignments are consistent with the calculations of previous studies.

According to Table 1 and in keeping with earlier Raman spectroscopic analysis (*14*), the 1710 cm^{-1} band is associated with the C_4═O carbonyl stretching motion and offers a means for following changes in the H-bonding interactions at the C_4═O group. The 1665 cm^{-1} band is associated with the C_2═O carbonyl stretching motion. Table 1 indicates that the 1625, 1550, and 1500 cm^{-1} bands are associated mainly with stretching vibrations of the isoalloxazine ring I and/or stretching vibrations of the C_{10a}−N_1 and N_5−C_{4a} that are coupled to the ring I mode. The 1580 cm^{-1} band is associated with the N_1−C_{10a}−C_{4a}−N_5 region and may assume importance in later studies because chemical changes occur in this region of the ring. The 1460 cm^{-1} band consists of highly coupled stretching motions of three rings, and is also coupled with methyl deformations. The most intense Raman band, near 1355 cm^{-1}, is made up of at least two different modes, each containing contributions from all three rings of the isoalloxazine. Near 1410 cm^{-1}, there are two bands, 1420 and 1400 cm^{-1}, that are associated with ring I and II (Table 1). The 1300−1000 cm^{-1} is a sensitive fingerprint region and is also quite congested. Most of the peaks in this region involve C_6−H, C_9−H, and N_3−H in-plane bending motions.

The C═O stretching region is unique in that changes can be interpreted fairly simply on the basis of environmental factors such as changes in hydrogen bonding. In contrast, the interpretation of the observed variation in the modes near 1500, 1410, and 1355 cm^{-1} with changes in state or solvent (Figure 2) cannot be put in simple terms. Our understanding of their properties is improving as quantum mechanical based calculations become increasingly sophisticated. At this point, however, we use the 1500, 1410, and 1355 cm^{-1} features as empirical markers to report on the flavin ring in different dielectric environments. Since both the 1260 and 1150 cm^{-1} bands have contributions from N_3−H bending (Table 1), the observed changes in these peaks in Figure 2 may be due, in part, to changes in H-bonding at N_3−H.

The Raman Markers for "In" and "Out" Conformations of the Flavin. In the wild-type enzyme and the substrate−PHBH complex, crystallographic studies demonstrate that the flavin is in the "in" conformation in which the isoalloxazine ring III forms extensive H-bonds with surrounding residues. The crystallographic data revealed that C_4═O forms two H-bonds with the peptide NHs of Gly46 and Val47 residues. When the 2,4-di-OH-benzoate binds to protein, the steric overlap between 2-OH group and the flavin pushes the flavin into the "out" conformation (*2*). In the protein, solvent molecules fill the space vacated by the isoalloxazine ring and also form H-bonds with the exposed flavin ring III. Thus, at C_4═O, for example, the peptide NH hydrogen

Table 1: Assignment of Flavin Raman Bands in the 1000−1750 cm^{-1} Region

raman bands (cm^{-1})	intensity	assignment
1720−1690	w ∼ m	νC_4═O, νC_2═O
1660−1690	w	νC_2═O, N_3−H bend
1630−1625	w ∼ m	ring I stretch
1585−1575	m ∼ s	νN_5-C_{4a}, $\nu C_{10a}-N_1$
1550−1540	m ∼ s	ring I stretch and $\nu C_{10a}-N_1$
1505−1500	m	νN_5-C_{4a}, $\nu C_{10a}-N_1$, and ring I stretch
(Here there are contributions from two bands near 1515 and 1495 cm^{-1})		
1465−1460	m	ring I, II, III + methyl deformation
1410−1400	m	Ring I, II, and methyl deformation
(Here there are contributions from two bands near 1415 and 1395 cm^{-1})		
1365−1340 (*three overlapping bands*)	very strong	a. Ring I, II, III stretch, and $N_{10}-C_{1'}$ stretch b. N_3−H bend c. Ring I, II, III stretch, and N_3−H bend
1305−1300	w	Ring I, II, III + C_6−H bend + N_3−H bend
1290−1280	w	Ring I, II, III + N_3−H bend
1265−1240	m	C_6−H bend + C_9−H bend + N_3−H bend + νN_5-C_5a + $\nu N_{10}-C_{1'}$ (in lumiflavin)
1235−1225	m ∼ s	C_6−H bend and C_9−H bend
1185−1175	m	C_6−H bend, C_9−H bend and N_3−H bend
1160−1150	m	C_6−H bend, N_3−H bend + Ring II
1140−1135	w	C_6−H, C_9−H bend and N_3−H bend
1115−1110	w	C_6−H bend + C_7−methyl C stretch + C_8−methyl C stretch
1080−1060	w ∼ m	C_7−methyl C stretch + C_8−methyl C stretch and Ring I, II, III bend

bonds are replaced by the hydrogen bonds with water molecules.

The Raman spectra of FAD bound to PHBH were generated by subtracting the spectrum of the apoenzyme from the spectrum of the holoenzyme. For PHBH complexes with substrate or substrate analogue, the Raman spectra of FAD in the complexes were obtained by recording the spectrum of apo-PHBH plus ligand, adding FAD to the complex, and subtracting the spectrum of the binary complex from that of the ternary complex. Figure 3 shows the Raman spectra of FAD in wild-type enzyme and in wild-type PHBH plus pOHB where the flavin is in the "in" form and in wild-type plus 2,4-di-OH-benzoate where the flavin is in the "out" conformation. The Raman spectra of the buried flavin show several differences when compared with the spectrum of the free flavin seen in Figure 2. For example, the stretching mode of the C_4═O shifts from 1712 cm^{-1} in the free form to 1700 cm^{-1} in the "in" form. This spectroscopic result indicates that the H-bond between C_4═O and protein in the active site is stronger than that to water in the case of free FAD.

Comparing the "in" and "out" forms, the most marked spectral differences are the bands near 1410 and 1355 cm^{-1}, associated with the ring modes of the isoalloxazine. In aqueous solution, each of these bands has usually been thought to exist as a separate single band, with the exception of glucose oxidase, where a possible doublet was seen near 1355 cm^{-1} (*23*). In the solid state, however, both the 1410 and 1355 cm^{-1} bands appear split in the FT-Raman spectrum of the lumiflavin (*24*). We recorded the 785 nm excited spectrum of solid lumiflavin seen in Figure 2A, and it confirms the FT-Raman data. Two features are observed at 1415 and 1396 cm^{-1}, and two peaks can be seen at 1361 and 1347 cm^{-1}. Our calculations indicate that there are at least two Raman active normal modes in both the 1410 and 1355 cm^{-1} regions (Table 1). The Raman spectrum of the "out" conformation of flavin in PHBH (Figure 3C, Figure 4C), is almost identical to that of the free FAD in aqueous

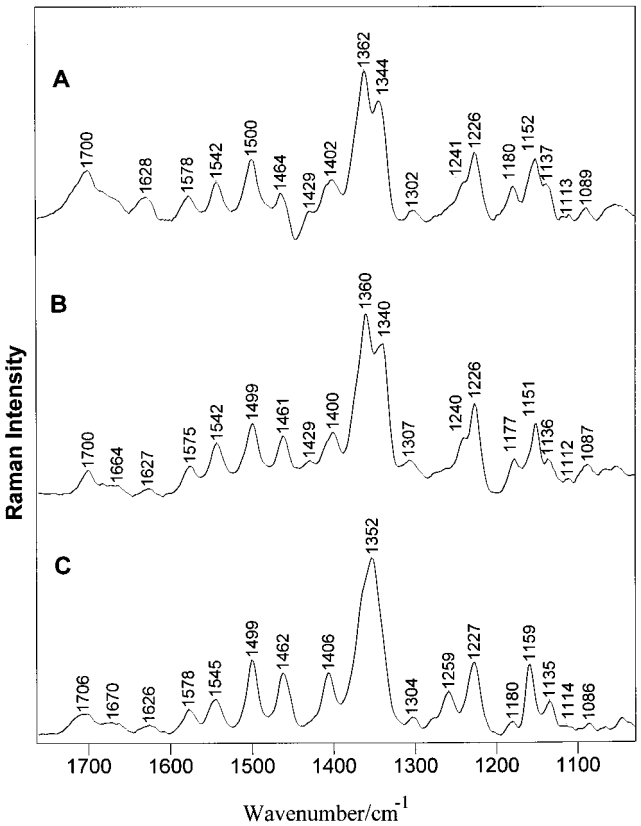

FIGURE 3: Raman difference spectra for FAD bound to WT-PHBH. (A) FAD in wild-type PHBH. (B) FAD in wild-type PHBH + pOHB. (C) FAD in wild-type PHBH + 2,4-di-OH-benzoate, 850 mW, 752 nm laser excitation, 10 min binning. Enzyme is 300 μM in 100 mM phosphate buffer, pH 7.5.

buffer (shown in Figure 2D). Single features are detected at 1410 and 1355 cm^{-1} for free FAD and, for the "out" conformer at 1406 and 1352 cm^{-1}, with a shoulder evident on the high wavenumber side of the latter feature. These similarities provide good evidence for the solvent exposed

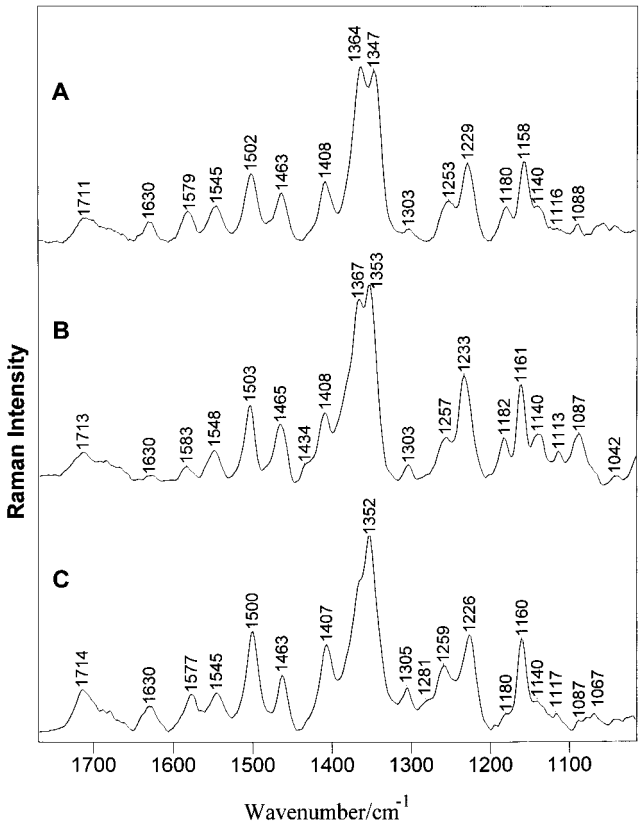

FIGURE 4: Raman difference spectra for FAD bound to Tyr222Phe−PHBH. (A) FAD in Tyr222Phe PHBH. (B) FAD in Tyr222Phe−PHBH + pOHB. (C) FAD in Tyr222Phe PHBH + 2,4-di-OH-benzoate; 850 mW 752 nm laser excitation, 10 min binning. Enzyme is 300 μM in 100 mM phosphate buffer, pH 7.5.

nature of the "out" form. However, when the flavin assumes the "in" conformation, (Figure 3A, 3B) two features are observed near 1400 and 1430 cm^{-1} and two features can be observed near 1360 and 1340 cm^{-1}. On the basis of the corresponding splitting seen in solid lumiflavin (Figure 2A), we ascribe the observed splitting for the "in" conformation to the flavin being held in a well-formed pocket from which water molecules are excluded.

There are some other significant Raman spectral differences between "in" and "out", such as the 1258 and 1160 cm^{-1} bands, that are "downshifted" in the "in" conformation compared to the "out" conformation. The underlying reason is likely the same as described above—the nonpolar environment making up the isoalloxazine ring-binding pocket shifts these bands to lower frequencies. In addition, it is likely that part of the observed shifts is due to a change in the H-bonding pattern at N_3−H, since both the 1258 and 1160 cm^{-1} bands have contributions from a bending motion of that moiety (Table 1). The Raman markers for "in" and "out" are summarized in Table 2.

Evidence for a Mixed Population of "In" and "Out" Conformations. The mutant enzyme Tyr222Phe played an important role in the discovery of the "out" form of the flavin in PHBH, since the crystal structure of the pOHB−Tyr222Phe complex clearly showed a preponderance of the "out" form (*6*, *7*). In the Tyr222Phe mutant, Entsch and van Berkel (*25*) ascribed the appearance of the "out" conformation to the removal of a strong H-bond that forms between Tyr222's OH group and the COO^- group of the substrate in

Table 2: The Raman Markers for the "In" and "Out" Conformations

complexes	conformation	Raman markers (cm^{-1})
WT−PHBH	in	1700, 1429, 1402, 1362, 1344, 1241, 1152
WT−PHBH/pOHB	in	1700, 1429, 1400, 1360, 1340, 1240, 1151
WT−PHBH/2,4-di-OHB	out	1706, 1406, 1352, 1259, 1159
Tyr222Phe−2,4-di-OHB	out	1714, 1407, 1352, 1259, 1160

the "in" complex. For the pOHB−Tyr222Phe complex crystallographic refinement suggested that a mixed population of "out" and "in" forms exist in the crystal, in the ratio of approximately 70:30, respectively (*6*). The Raman spectrum of this complex, shown in Figure 4B, suggests that a similar situation occurs in the solution phase. The band at 1408 cm^{-1} includes a major "out" population, but the shoulder near 1430 cm^{-1} suggests that this is accompanied by a minor "in" population. The relative intensity of the band at 1367 cm^{-1} is less than seen for 100% "out" in Figure 4C, suggesting a mixed population. Furthermore, compared to Figure 3C the "out" marker at 1257 cm^{-1} is less well-resolved from the 1233 cm^{-1} band, consistent with the presence of an "in" population contributing near 1240 cm^{-1}. Thus, the data for the Raman markers for the pOHB−Tyr222Phe complex in solution are consistent with the finding for the crystallized complex.

The spectral data for Tyr222Phe PHBH, in the absence of substrate (Figure 4A), are very similar to those in Figure 3C. No crystal data are available for the enzyme, but based on the Raman data we predict that the flavin is predominantly in the "out" conformation. In the complex involving Tyr222Phe PHBH with the substrate analogue 2,4 di-OH-B, the Raman data (Figure 4C) are very similar to those for the complex of wt enzyme with the same substrate analogue (Figure 3C). This points to the presence of only an "out" population with no evidence for an "in" species.

FUTURE APPLICATIONS

The demonstration that the "in" and "out" forms of the flavin ring on PHBH have distinctive Raman marker bands provides a number of opportunities. The marker bands can be used to determine the environment of the ring in a PHBH complex that has not been analyzed, or has resisted analysis, by X-ray methods. For complexes that contain a mixed population of "in" and "out" forms, band shape analysis and deconvolution in the marker regions should provide at least a semiquantitative estimate of the relative amounts of the two forms. Carrying out this analysis over a range of temperatures has the potential to determine enthalpic and entropic differences for the two conformers. In light of the possibility that the "in" and "out" forms catalyze chemical steps at different points on the reaction pathway, knowledge of these thermodynamic values may be necessary for a comprehensive understanding of mechanism.

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