that the reverse reactions may well represent a plausible path for methanol synthesis on Ru(001):

$$CH_3OH(g) \rightarrow CH_3O(a) + H(a)$$

$$CH_3O(a) \rightarrow \eta^2\text{-}H_2CO(a) + H(a)$$

$$\eta^2\text{-}H_2CO(a) \rightarrow \eta^2\text{-}HCO(a) + H(a)$$

$$\eta^2\text{-}HCO(a) \rightarrow CO(a) + H(a)$$

It is interesting to note that a reaction sequence analogous to the reverse of the above set of reactions has been postulated to explain the formation of methanol from CO and hydrogen by $Ru_3(CO)_{12}$ under homogeneous reaction conditions.[17] Our observations should be interpreted as lending support to this proposition.

V. Summary

The important conclusions of this work may be summarized as follows: 1. Initial adsorption of formaldehyde on the clean Ru(001) surface at 80 K is dissociative, producing adsorbed CO and hydrogen. As binding sites for hydrogen adatoms become occupied, the total decomposition is inhibited, leading first to the formation of η^2-formyl and then to molecular adsorption of formaldehyde in both η^1 and η^2 configurations. 2. Heating of the clean surface after a monolayer saturation exposure to formaldehyde at 80 K causes the η^1-formaldehyde to desorb molecularly and the η^2-formaldehyde to decompose (to adsorbed CO and hydrogen). The clean Ru(001) surface is active for the decomposition of approximately a quarter-monolayer of formaldehyde under these conditions. 3. The presence of a p(2×2) oxygen overlayer on the Ru(001) surface increases the Lewis acidity of the surface ruthenium atoms, and adsorption of formaldehyde at 80 K on the Ru(001)-p(2×2)O surface produces vibrational spectra characteristic of η^1-formaldehyde for all submonolayer coverages. 4. Heating of the Ru(001)-p(2×2)O surface after a monolayer saturation exposure to formaldehyde at 80 K causes the η^1-formaldehyde to desorb molecularly, with some conversion to η^2-formaldehyde. In addition to decomposition of the η^2-formaldehyde to CO and hydrogen, reaction of η^1-formaldehyde with the oxygen adatoms of the p(2×2)O overlayer to produce η^2-formate occurs between 150 and 300 K. The Ru(001)-p(2×2)O surface is active for approximately one-half as much formaldehyde decomposition (via both η^2-formaldehyde and η^2-formate intermediates) as the clean surface under these conditions. 5. The appearance of η^2-formyl and η^2-formaldehyde as stable intermediates in the decomposition of formaldehyde on Ru(001) supports a reaction mechanism for CO hydrogenation to methanol under heterogeneous conditions which includes these species as important intermediates.

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Registry No. HCHO, 50-00-0; CO, 630-08-0; Ru, 7440-18-8.

Substrate Effect on Flavin-Enzyme Interaction in *p*-Hydroxybenzoate Hydroxylase as Probed by Resonance Inverse Raman Spectroscopy

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Abstract: Binding between the FAD and apoprotein of *p*-hydroxybenzoate hydroxylase (E.C. 1.14.13.2) from *Pseudomonas fluorescens* has been examined by using resonance inverse Raman spectroscopy. The vibrations of the flavin chromophore have been studied in the presence of substrates (*p*-hydroxybenzoate, 2,4-dihydroxybenzoate, and *p*-aminobenzoate), inhibitors (chloride and azide), and an effector (6-hydroxynicotinate). Ternary systems involving enzyme, inhibitor, and substrate were also examined. The 1195- and 1418-cm^{-1} bands are significantly shifted in frequency upon binding either substrate, inhibitor, or effector. The 1163-, 1311-, and 1595-cm^{-1} bands shifted in the presence of inhibitor, effector, or in the ternary complex, but not in the presence of substrate, alone. The 1184-cm^{-1} band was affected by azide and 6-hydroxynicotinate. The 1241-cm^{-1} band was perturbed in the presence of 2,4-dihydroxybenzoate. Both the 1241- and 1258-cm^{-1} bands were shifted in the ternary complexes and in the presence of 6-hydroxynicotinate. The 1284-cm^{-1} band was shifted in azide. The intense 1355/1370-cm^{-1} band was unchanged. The 1563-cm^{-1} band in the free enzyme was unchanged by *p*-hydroxybenzoate, chloride, 6-hydroxynicotinate, and azide plus 2,4-dihydroxybenzoate but was shifted to higher wavenumbers in azide and did not appear in *p*-aminobenzoate, 2,4-dihydroxybenzoate, and azide plus *p*-hydroxybenzoate. These changes in flavin vibrational frequencies reflect conformational changes in the enzyme upon binding ligands. Hydrogen bonding between FAD at N(1), C(2)=O, N(3), C(4)=O, and amino acid chains 45-47 and 299-300 was strengthened by binding inhibitors, effectors, and substrates causing the shifts seen in the Raman spectra.

p-Hydroxybenzoate hydroxylase (E.C. 1.14.13.2) is an NADPH dependent flavoenzyme which hydroxylates *p*-hydroxybenzoic acid to form 3,4-dihydroxybenzoate.[1] The reaction is a two-step process in which molecular oxygen is consumed.[1-3] In the first step, the oxidized enzyme combines with *p*-hydroxybenzoate and NADPH, then the bound FAD is reduced by NADPH. In the second step, the reduced enzyme-substrate complex reacts with molecular oxygen to yield a flavin-activated oxygen species. The substrate is hydroxylated, the oxidized FAD is regenerated. The complexes investigated in this work all involve oxidized enzyme and either substrate, effector, or inhibitor. They are made in the absence of the reducing agent NADPH and as such are intrinsically stable.

There have been many kinetic studies of the reactions catalyzed by this enzyme.[1-6] The crystal structure of the enzyme-*p*-

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hydroxybenzoate complex has been determined.[7-10] A variety of effectors have been found.[1,2] Effectors are compounds which increase the NADPH oxidation rate, but which themselves are not hydroxylated. Enzymatic inhibitors have also been identified.[2,3,5]

One question of current interest is the mechanism by which substrates increase the rate of FAD reduction by NADPH, by as much as 40 000-fold.[4] It is generally accepted that substrate binding changes the conformation of the active site. With Raman spectroscopy as a tool, we have examined the effect of substrate binding on the environment of the FAD in the active site and compared these effects with those resulting from effector binding. Another question involves the effect of monovalent anion inhibitors on the active site. These inhibitors bind to the enzyme and dramatically reduce the rates of the oxidative half of the reaction.[2] We have used Raman spectroscopy to more clearly define changes in the active site structure which could account for this inhibition.

In this study, we have used inverse Raman spectroscopy, a nonlinear Raman technique, to probe the interaction between the FAD and surrounding protein in the presence of a variety of substrates (*p*-hydroxybenzoate, 2,4-dihydroxybenzoate, and *p*-aminobenzoate), inhibitors (azide and chloride), and an effector (6-hydroxynicotinate). We have also examined two ternary systems with enzyme, inhibitor, and substrate present (enzyme + azide + 2,4-dihydroxybenzoate and enzyme + azide + *p*-hydroxybenzoate).

Resonance Raman spectroscopy is a useful technique for probing changes in hydrogen bonding between a chromophore and its surroundings in an enzymatic system. Conformational changes in the protein chain induced by binding ligands can be inferred from changes in Raman frequencies and intensities of the chromophore itself. Raman spectroscopy and nonlinear Raman techniques (CARS and IRS) have been applied extensively to study the environment of flavins in flavoproteins.[11-16] Many of the vibrational bands for the free flavin spectrum have been assigned and an approximate normal coordinate analysis of the flavin chromophore has been presented.[17] The band numbering system defined in that work is commonly used to describe flavin Raman spectra.

Two groups have attempted to define criteria for identifying the strength of hydrogen bonding to the flavin.[14,16] This work focused on the isoalloxazine portion of the flavin, in which there are three good hydrogen bond acceptors and one good hydrogen bond donor. The acceptors are N(1) and the carbonyl oxygens at C(2) and C(4). The donor is the hydrogen at N(3). Both groups draw conclusions primarily from bulk solvent effects. That is, their criteria are based on changes in band positions and intensities in Raman spectra of flavins in a series of solvents.

Schmidt and co-workers[16] propose that strengthened hydrogen bonding is indicated by several criteria. These include a decrease in the frequency of band II (ca. 1580–1590 cm^{-1}), an increase in the frequency of band X (ca. 1250–1260 cm^{-1}), and the presence of band IX (ca. 1285 cm^{-1}). Muller and co-workers[14] suggest somewhat different criteria. They argue that strengthened hydrogen bonding is indicated by a shift to higher frequency of bands XII, XI, VI, and I. They state that band II is unaffected.

Application of these solvent study correlations to flavoproteins is difficult. The criteria themselves depend upon the choice of both model compounds and solvents. In addition, perturbation of the flavin environment in a protein may cause changes in all or only some hydrogen bonds. The effect on the spectrum can be different from the gross changes induced by change of solvent.

Flavin bands with a large component of N(3)–H stretch are expected to shift to higher wavenumber as hydrogen bonding at this position increases. This effect has been previously observed in band X.[16] Bands which are primarily stretching modes of the isoalloxazine skeleton may be affected in more subtle ways, depending on the potential energy distribution of the vibration and upon the site of hydrogen bonding.

While there is now a large body of flavin resonance Raman spectra. the theoretical interpretation is less well advanced.[17] Because of the lack of a rigorous assignment of the spectrum, our own interpretation of ligand binding effects must be regarded as tentative.

The greatest experimental problem in flavin Raman spectroscopy is that free flavins and many flavoenzymes are fluorescent. Several Raman spectra of free flavins have been obtained by using KI as a collisional fluorescence quencher.[18] Raman spectra of free flavins have also been obtained by binding the flavins to a protein, such a riboflavin binding protein to quench fluorescence.[19] Coherent antistokes Raman spectroscopy (CARS) and inverse Raman spectroscopy (IRS) are coherent Raman techniques which discriminate against fluorescence and make it possible to study a wide variety of free flavins and flavoenzymes without alteration. CARS has been applied extensively to study both free flavins and flavoproteins.[12,14,15] Using inverse Raman spectroscopy, we have studied several fluorescent flavins, such as 8-amino- and 8-hydroxyriboflavin,[20] flavoenzymes including lactate oxidase,[21] and old yellow enzyme.[22] We have obtained significant structural information from spectra unobscured by fluorescence.

Experimental Section

Preparation. *p*-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* was prepared as previously described by Muller et al.[23] and stored as an ammonium sulfate precipitate at 4 °C until used. Immediately prior to use, the enzyme was transferred to a 50 mM potassium phosphate buffer, pH 6.55, containing 0.3 mM EDTA and a saturating concentration of the ligand of interest. The final enzyme concentration was 0.25 mM in all cases. Nine different cases were studied: *p*-hydroxybenzoate hydroxylase alone; enzyme + *p*-hydroxybenzoate (substrate); enzyme + 2,4-dihydroxybenzoate (substrate); enzyme + *p*-aminobenzoate (substrate); enzyme + chloride (inhibitor); enzyme + azide (inhibitor); enzyme + 6-hydroxynicotinate (effector); enzyme + azide + *p*-hydroxybenzoate; and enzyme + azide + 2,4-dihydroxybenzoate.

Spectroscopic Measurements. The inverse Raman experimental apparatus used in these experiments was a modification of an earlier de-

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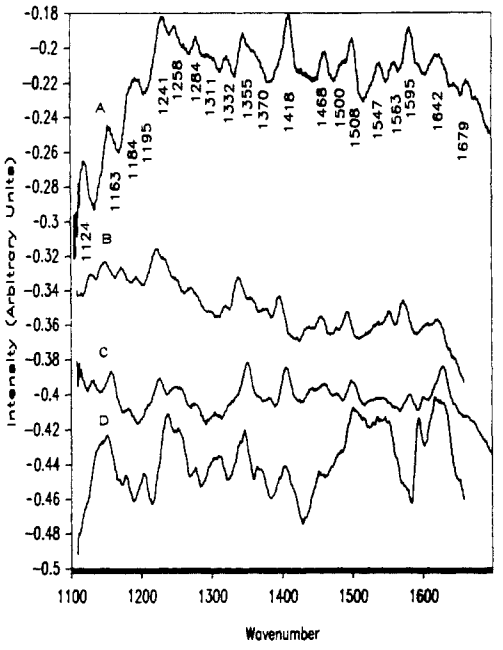

Figure 1. Resonance inverse Raman spectra of *p*-hydroxybenzoate hydroxylase and substrates: (A) enzyme, (B) enzyme + 5 mM *p*-hydroxybenzoate, (C) enzyme + 10 mM 2,4-dihydroxybenzoate, and (D) enzyme + 5 mM *p*-aminobenzoate. The enzyme was buffered at pH 6.55 in 50 mM potassium phosphate + 0.3 mM EDTA. A saturating concentration of each ligand was used. The final enzyme concentration was 0.25 mM in all cases. Each spectrum was normalized to an external 1 M NO_3^- standard. The laser excitation frequency was 514.5 nm.

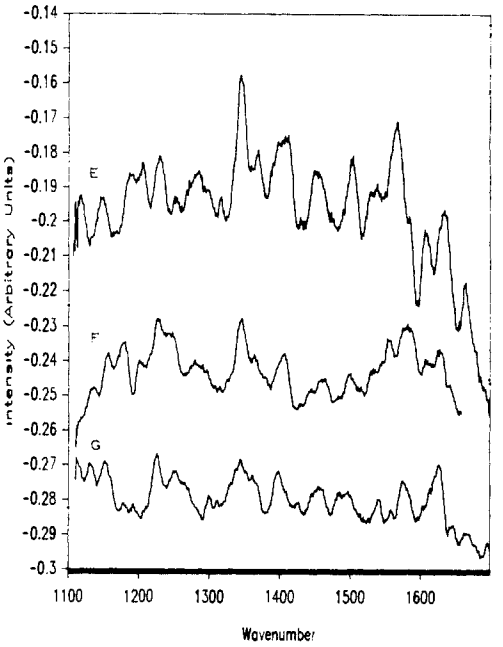

Figure 2. Resonance inverse Raman spectra of *p*-hydroxybenzoate hydroxylase and inhibitors and an effector: (E) enzyme + 40 mM azide, (F) enzyme + 40 mM chloride, and (G) enzyme + 10 mM 6-hydroxynicotinate.

sign.[24] The argon ion (Lexel 85-1) 514.5-nm (19436 cm^{-1}) line was used as the pump beam. This wavelength provided resonance Raman enhanced spectra. The pump beam power was 100–150 mW. The probe beam was an XeF excimer laser (351 nm) pumped dye laser (Tachisto 800 XR, Molectron DL-19). The excimer laser was operated at 13 pulses per s, with an energy of 60–90 mJ per pulse. Coumarin 540 was used as the laser dye and was scanned from 542 nm ($\Delta\omega$ = 1100 cm^{-1}) to 567 nm ($\Delta\omega$ = 1740 cm^{-1}). The two beams were combined colinearly. The

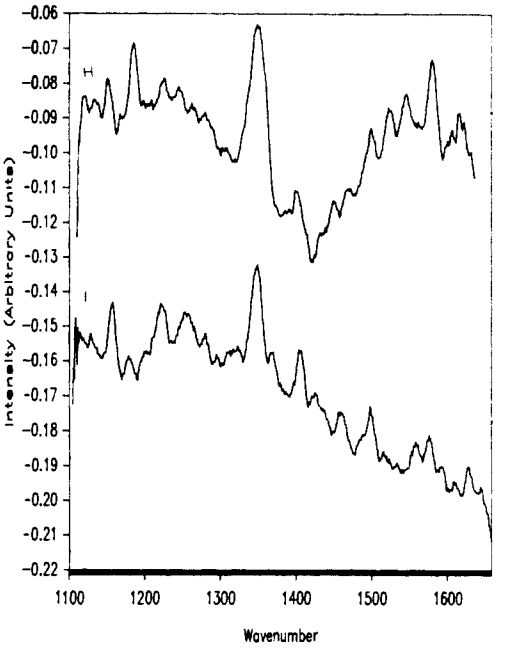

Figure 3. Resonace inverse Raman spectra of *p*-hydroxybenzoate hydroxylase in ternary complexes: (H) enzyme + 5 mM *p*-hydroxybenzoate + 40 mM azide and (I) enzyme + 10 mM 2,4-dihydroxybenozate + 80 mM azide.

sample cell was a 20-mm path length, 1 mm wide, rectangular glass microcell centered at the focal point of the laser beams. A diffraction grating was used to separate probe and pump beams. The signal was detected as a 1% induced absorption from the argon ion laser by an ac coupled photodiode and amplifier.[24,25] Scanning of the dye laser and data acquisition was controlled with a Digital Equipment Corp. PDP-11/03 microcomputer.[26]

Resonance inverse Raman band shapes are non-Lorentzian.[27,28] Raman frequencies do not correspond to experimental peak maxima. The correct Raman frequency assignment for each band was found by comparison of experimental bands to simulated bands, using S^0_0 and Γ_e evaluated[29,30] from the absorption spectrum for each system studied. The half-width at half-height of the S^0_0 transition was taken as the nominal electronic damping constant. The Raman damping constant was set to 5 cm^{-1}.

Results and Discussion

Figures 1, 2, and 3 illustrate the Raman spectra of *p*-hydroxybenzoate hydroxylase alone and in the presence of various ligands. The 1124-, 1163-, 1184-, 1195-, 1241-, 1258-, 1311-, 1418-, 1547-, 1563-, 1595-, and 1642-cm^{-1} bands of the free enzyme are shifted in frequency upon the addition of substrate, effectors, and inhibitors. The flavin ring numbering system defined in structure I will be used to describe the details of these changes.

Quite regular patterns are observed in the Raman spectral changes, if one compares the effects of substrates (*p*-hydroxybenzoate and 2,4-dihydroxybenzoate), inhibitors (azide and chloride), effector (6-hydroxynicotinate), and ternary complexes containing inhibitor and substrate. With few exceptions, changes in the frequency of a given band occur in the same direction for all of the complexes. In general, the changes in the Raman spectrum indicate a strengthening of hydrogen bonds between the flavin and its matrix upon addition of any ligand.

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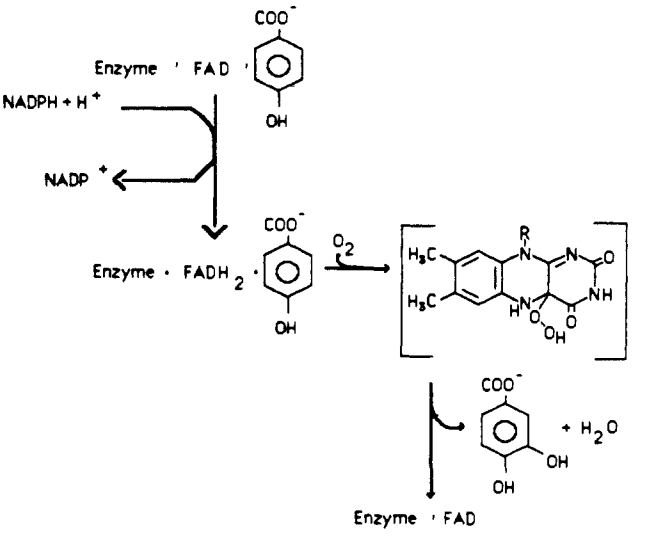
Table I. Flavin Raman Bands in *p*-Hydroxybenzoate Hydroxylase in the Presence of a Variety of Substrates, Effectors, and Inhibitors

band	enzyme alone	enzyme + *p*-hydroxybenzoate	enzyme + 2,4-dihydroxybenzoate	enzyme + azide	enzyme + chloride	enzyme + 6-hydroxynicotinate	enzyme + *p*-aminobenzoate	enzyme + azide + *p*-hydroxybenzoate	enzyme + azide + 2,4-dihydroxybenzoate
	1124	1144	1134		1137	1134			
XIII	1163	1163	1163	1155	1163	1163	1155	1152	1163
XII	1184 (sh)	1187	1184	1189	1184	1179	1184	1189	1184
	1195	1205		1208	1208	1195	1211	1226	
XI	1241	1241	1232	1241	1241	1237	1246	1253	1232
X	1258	1258 (sh)	1258 (wide)	1258	1258 (sh)	1263 (wide)	1261	1268	1268
IX	1284	1284	1284	1289 (wide)	1284		1284	1284	1284
VIII	1311 (sh)	1311	1311	1316			1316	1311	
	1332	1332							
VII	1355, 1370 (sh)	1355, 1370 (sh)	1357	1355, 1368	1355, 1370	1355, 1370	1355, 1370	1355	1355, 1370
VI	1418	1404	1411	1418 (wide)	1411	1405	1411	1405	1405
V	1468	1453 (sh), 1468	1445, 1468, 1479	1453 (wide)	1442 (sh), 1468	1466 (wide)	1458	1453	1468
IV	1500 (sh), 1508	1500 (sh), 1508	1508	1508	1508	1500, 1508	1508	1503, 1526	1500, 1508
III	1547	1547 (sh)	1547 (wide)	1547	1537 (sh)	1547	1547	1547	
	1563	1563		1574	1563	1563			1563
II	1595	1595	1595		1595 (wide)	1584	1600	1587	1584
		1621		1611	1611				
I	1642	1642	1632	1637	1642	1626	1637	1629	1632
	1679			1663		1679			
S_0^0 cm^{-1}	21053	20921	20877	21008	21008	20833	21053	20964	21008



structure I

Intensity changes in bands associated with ring III are also apparent. We identify the bands by their frequencies in free enzyme. Throughout this paper, we equate shifts in band position with changes in hydrogen bonding between the flavin and protein[18] and changes in peak intensity with alterations in ring stacking interactions between the flavin and aromatic amino acid residues[31] (known to be in the vicinity of the flavin.[9,10]

1124-cm^{-1} Band. The 1124-cm^{-1} band is unassigned in the flavin normal coordinate analysis.[17] It has been previously reported in the spectrum of free FMN.[12] It corresponds closely in frequency to the 1138-cm^{-1} band seen in N(3)–D riboflavin bound to RBP[13] and to the 1147-cm^{-1} band reported in the preresonant Raman spectrum of FAD itself.[35] We take these observations to indicate substantial N(3)–H bend contribution to this band.

The shifts from 1124 cm^{-1} in the free enzyme to 1144 cm^{-1} in the enzyme-*p*-hydroxybenzoate complex and to 1134 cm^{-1} in the 2,4-dihydroxybenzoate complex suggest that hydrogen bonding to the flavin at N(3) may be strengthened by the presence of substrate. The increase in frequency of this band to 1137 cm^{-1} in the presence of chloride and to 1134 cm^{-1} in the presence of 6-hydroxynicotinate indicates that these compounds may also strengthen hydrogen bonding at N(3).[9,10]

1163-cm^{-1} Band. This band shifts to 1155 cm^{-1} on binding azide and *p*-aminobenzoate and to 1152 cm^{-1} in the ternary complex involving azide + *p*-hydroxybenzoate. This band is not sensitive to solvation effects in free flavins.[16] But, Kitagawa et al. have shown that the band is sensitive to N(3) deuteration but not to ^{15}N(3) substitution. It is perturbed by isotopic substitution at positions C(4), C(4a), and C(10a) of the flavin.[13] Normal mode analysis suggests involvement of $\nu(C_{4a}–C_4)$ and $\nu(C_{4a}–C_{10a})$.[17] In view of this complexity, prediction of hydrogen bonding effects is not possible. We note only that azide and *p*-aminobenzoate alter hydrogen bonding between the surrounding amino acid residues and C(4)=O or possibly N(3)–H of the flavin.

1184-cm^{-1} Band. Small changes are observed in the 1184-cm^{-1} band. It shifts to 1187 cm^{-1} upon binding *p*-hydroxybenzoate and to 1189 cm^{-1} upon binding azide and azide + *p*-hydroxybenzoate. This band shifts down to 1179 cm^{-1} when the enzyme binds 6-hydroxynicotinate. The 1184-cm^{-1} band is a delocalized ring III mode according to the normal coordinate analysis.[17] It is affected by isotopic substitution at C(2), C(4a), N(1,3), and

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N(5).[13] The shift in frequency of this band is a general, but insensitive, indication of change in hydrogen bonding to ring III.

1195-cm^{-1} Band. This band is not assigned in the normal coordinate analysis[17] and not generally seen. A band in this vicinity is observed in the spectrum of the N(3)–D form of riboflavin in RBP[13] at 1209 cm^{-1}, which shifts to 1206 cm^{-1} on substituting ^{13}C at C(2) and to 1205 cm^{-1} on substituting ^{15}N at N(1,3). We, therefore, suggest that our 1195-cm^{-1} band is associated with N3) and C(2). It shifts from 1195 cm^{-1} in the free enzyme to 1205 cm^{-1} upon addition of *p*-hydroxybenzoate, to 1208 cm^{-1} upon addition of azide and chloride, to 1211 cm^{-1} upon addition of *p*-aminobenzoate, and to 1226 cm^{-1} in the azide + *p*-hydroxybenzoate ternary complex. The progressive shift to longer wavenumber suggests a trend of increasing strength of hydrogen bonding to N(3) or C(2)=O in the presence of *p*-hydroxybenzoate, azide, chloride, *p*-aminobenzoate, and azide + *p*-hydroxybenzoate.

1241-cm^{-1} Band. The 1241-cm^{-1} band is shifted down to 1232 cm^{-1} in the enzyme–2,4-dihydroxybenzoate complex and to 1237 cm^{-1} in the 6-hydroxynicotinate complex. The band shifts in opposite directions in the two ternary complexes, up to 1253 cm^{-1} in azide + *p*-hydroxybenzoate and down to 1232 cm^{-1} in azide + 2,4-dihydroxybenzoate. This is the only band in which the shifts, relative to the free enzyme, are not all in the same direction for each ligand.

We correlate this band with the 1229-cm^{-1} band in riboflavin bound to RBP. That band is slightly sensitive to N(3) deuteration and to isotopic substitution at C(4a), N(1), and N(3).[13] The reasons for the large shifts are unclear at present.

1258-cm^{-1} Band. In our systems, only 6-hydroxynicotinate and the ternary complexes cause this band to shift. Large changes, to 1268 cm^{-1}, are seen only with the ternary complexes. This band is commonly used as an indicator of changes in hydrogen bonding at N(3).[13,14,16] In riboflavin, the band moves to higher wavenumbers on deuteration of N(3).[19] The 1258-cm^{-1} band is affected by ^{13}C and ^{15}N substitution only at C(2), N(1), and N(3). It is likely that the band responds to changes at N(3) and C(2)=O. The insensitivity of this band to addition of substrates *p*-hydroxybenzoate or 2,4-dihydroxybenzoate and inhibitors azide or chloride suggests that with those ligands hydrogen bond changes at N(3) may not be large. In the other systems the increase in frequency is an unambiguous indication of strengthened hydrogen bonding.

1284-cm^{-1} Band. The 1284-cm^{-1} band is widened and shifts only upon addition of azide. This band is assigned to a δ CH_3 (ring I) in the normal coordinate analysis[17] and is sensitive to isotopic substitution at C(4a).[13] It is not expected to be sensitive to hydrogen bonding.

1311-cm^{-1} Band. The 1311-cm^{-1} band appears as a shoulder in the free enzyme spectrum. It is shifted to 1316 cm^{-1} in the azide and *p*-aminobenzoate spectra and is not seen in chloride, 6-hydroxynicotinate, or azide + 2,4-dihydroxybenzoate. This band is affected by isotopic substitution at C(4a) and/or N(5).[13]

1332-cm^{-1} Band. This weak band appears only in the free enzyme and in the enzyme + *p*-hydroxybenzoate. It is not assigned in the normal coordinate analysis, and no band has been seen at this frequency for riboflavin in RBP.[17]

1355/1370-cm^{-1} Band. In free flavins the most flavoprotins, a single intense band is found in the flavin spectrum around 1355 cm^{-1}. In *p*-hydroxybenzoate hydroxylase, this band is split. The positions of these bands are unaffected by ligand binding. The basis for this splitting, and its significance, is unclear, but it has been previously noted in glucose oxidase.[13] In lactate oxidase addition of some ligands resolved this band into two bands.[21]

1418-cm^{-1} Band. The 1418-cm^{-1} band of free enzyme shifts to 1404 cm^{-1} in enzyme + *p*-hydroxybenzoate. Similarly, this band shifts to 1405 cm^{-1} in 6-hydroxynicotinate, azide + *p*-hydroxybenzoate, and azide + 2,4-dihydroxybenzoate. In enzyme + 2,4-dihydroxybenzoate, enzyme + chloride, and enzyme + *p*-aminobenzoate this band shifts to 1411 cm^{-1}. This band is most affected by isotopic substitution at C(2), C(4), C(4a), and N1,3).[13] It has been assigned[17] to ν_{22}, $\nu(N_1–C_2)$, $\nu(C_{5a}–C_6)$, $\nu(C_8–C_9)$, $\nu(C_{5a}–C_{9a})$ mode. Dutta[11] saw a 10-cm^{-1} shift of this band in FAD to 1406 cm^{-1} on N(3) protonation. This band is therefore a ring III mode responding to a general strengthening of hydrogen bonds in the presence of the ligands studied.

1468-cm^{-1} Band. There are differences in resolution of this band upon addition of ligand rather than any shifts. This can be attributed to changes in hydrogen bonding. Isotopic shift studies indicate a general sensitivity of this band to substitutions in rings II and III.[13] The normal coordinate analysis suggests this is a delocalized vibration.[17]

1500/1508-cm^{-1} Band. The 1500-cm^{-1} band appears only as a shoulder in the free enzyme, enzyme + *p*-hydroxybenzoate, 6-hydroxynicotinate, and in the enzyme plus azide and 2,4-dihydroxybenzoate. The 1508-cm^{-1} band does not change in position in any of the samples, except in the azide + *p*-hydroxybenzoate + enzyme, where a band is found at 1503 cm^{-1}. Normal mode analysis suggests ring wide contributions for this band, in a manner similar to the 1468-cm^{-1} band.

1547-cm^{-1} Band. This weak band shifts to 1537 cm^{-1} upon addition of chloride and is not visible in the azide + enzyme + 2,4-dihydroxybenzoate ternary complex. This band shifts 21 cm^{-1} on isotopic substitution at C(2,4,4a,10a) and only cm^{-1} on isotopic substitution at N(1,3,5).[13] It has been assigned to $\nu(C_{4a}–N_5)$ and to $\nu(C_{10a}–N_1)$.[17]

1563-cm^{-1} Band. This weak band is not seen in the enzyme + 2,4-dihydroxybenzoate, azide + *p*-hydroxybenzoate ternary complex, or enzyme + *p*-aminobenzoate samples. It is shifted to 1574 cm^{-1} in the enzyme + azide. It is not reported in the normal coordinate analysis[17] nor in the isotopic substitution study,[13] though a comparable band has been reported for several other flavoproteins.[12,15]

1595-cm^{-1} Band. This band undergoes major changes only in the ternary complexes and in the presence of 6-hydroxynicotinate. The changes are to lower frequency, consistent with strengthened hydrogen bonding[16] and with the pattern observed in band X (1258 cm^{-1}). The band is a ring II–III mode, sensitive to N(5), C(2), and C(4a) isotopic substitution.[13] We do not report its position in our azide spectrum, since the band is poorly resolved from the 1568-cm^{-1} band.

1642-cm^{-1} Band. The 1642-cm^{-1} band is a benzene ring I mode.[17] However, it has been shown that this band is weakly affected by isotopic substitution at C(2,4,4a,10a) and N(1,3,5).[13] The shifts seen here may be indicative of an indirect effect of hydrogen bond changes influencing the benzene vibration. This band shifts to 1632 cm^{-1} in enzyme + 2,4-dihydroxybenzoate and enzyme + azide + 2,4-dihydroxybenzoate, to 1626 cm^{-1} in enzyme + 6-hydroxy nicotinate, to 1629 cm^{-1} in enzyme + azide + *p*-hydroxybenzoate, and to 1637 cm^{-1} in enzyme + azide and enzyme + *p*-aminobenzoate. Splitting of this band is seen in chloride and azide samples where a second band is observed at 1611 cm^{-1}. In enzyme + *p*-hydroxybenzoate, a second band at 1621 cm^{-1} is observed. This could be due to resolution of the wide 1642-cm^{-1} band.

1679-cm^{-1} Band. The 1679-cm^{-1} band is seen in the free enzyme and in the enzyme + 6-hydroxynicotinate sample. A band is at 1663 cm^{-1} in the enzyme + azide sample. Both are probably carbonyl stretches and are reported occasionally.[14,35,36] These bands are weak and poorly characterized.

Intensity Changes. Tentative interpretations of relative band intensities are proposed. We cannot obtain complete Raman excitation profiles, so rigorous interpretations are not possible. Therefore, it is only possible to suggest interpretations of trends in relative intensities.

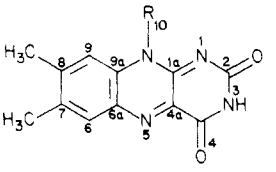
All sample spectra (Figures 1, 2, and 3) were normalized to the 1050-cm^{-1} mode of an external 1 M KNO_3 standard. The intensity scale of Figure 1 is twice that of Figures 2 and 3. Comparing the data in Figures 1, 2, and 3, the ratio of the intensity of the bands in the 1450–1600-cm^{-1} region can be seen relative to the 1355-cm^{-1} band change. In the cases of the free enzyme, the enzyme plus substrates, and the enzyme plus effector, the ratio

(36) Sugiyama, T.; Nisimoto, Y.; Mason, H. S.; Loehr, T. M. *Biochemistry* **1978**, *24*, 3012–3019.

is approximately 1:1. In the ternary complexes and the enzyme plus inhibitor samples the ratio appears to be much less than 1:1. A similar observation of intensity changes has been previously noted in lactate oxidase.[21] Those changes were attributed to alternations in the stacking between the flavin and an aromatic amino acid residue. No changes in Raman band positions were found in lactate oxidase. In *p*-hydroxybenzoate hydroxylase, however, changes are seen in band positions, indicating the possibility that there are differences between the ground-state and excited-state flavin configuration. This complicates interpretation of relative intensity measurements. However, we suggest that the decrease in intensity ratio in the enzyme plus inhibitors and the ternary complexes is due to a ring stacking interaction between the flavin and an aromatic residue.

There also appear to be changes in the intensity of the 1642-cm^{-1} band relative to the 1355-cm^{-1} band. In the presence of azide, 2,4-dihydroxybenzoate, *p*-aminobenzoate, and 6-hydroxynicotinate the 1642-cm^{-1} band appears larger. In the ternary complexes it appears to be smaller, and in *p*-hydroxybenzoate and chloride samples it is the same size. This band is also split in the *p*-hydroxybenzoate, chloride, and azide cases.

There are significant intensity differences between the enzyme alone and enzyme plus inhibitors. Changes in intensity are probably due to changes in position of aromatic residues near the flavin–substrate active site.[9,10]



structure II

Conclusions

p-Hydroxybenzoate hydroxylase is one of the few flavoenzymes for which both a crystal structure and an intensive Raman study have been completed. The crystal structure and Raman information are complementary and together can provide a more complete structural and dynamic picture of the enzyme.

By the criteria of Schmidt et al.,[16] the strength of hydrogen bonding between the flavin and the protein in unliganded *p*-hydroxybenzoate hydroxylase is weaker than is found for FMN in water.[12] Band X is at higher frequency, and band II is at lower frequency in free FMN.

From the crystal structure of the enzyme plus *p*-hydroxybenzoate complex,[9,10] it is known that ring III of the flavin is hydrogen bonded at N(1) to leucine 299, at C(2)=O to asparagine 300, and at both N(3) and C(4)=O to valine 47. Amino acid residues 299 and 300 are in the first turn of α-helix 5 in the protein. The dipole moment of this helix is oriented such that the positive pole is directed at the N(1)–C(2) region of the flavin. In addition, hydrophobic interactions at the dimethylbenzene end of the flavin involving tryptophan 185 and tyrosine 222 are indicated.[34] The substrate, *p*-hydroxybenzoate, binds near the N(5) position of the flavin with the plane of its phenyl ring oriented approximately perpendicular to the flavin plane and parallel to a sheet of β structure (B3) which forms one wall of the active site.[34] NADPH appears to bind in a cavity on the opposite side of the flavin, near α-helix 5. This cavity can bind negative ions and may also be the site for azide and chloride binding.[9,10]

p-Hydroxybenzoate is secured into a hydrophobic region of the active site via a salt bridge between its carboxyl group and arginine 214 and via hydrogen bonds between its hydroxyl group and tyrosines 201 and 385.[9,10,34] In the absence of *p*-hydroxybenzoate, it is expected that these amino acid side chains would find alternate hydrogen bonding sites which would change the structure of the active site. Consistent with this expectation, there are clear shifts in the 1124-, 1195-, and 1418-cm^{-1} bands of the Raman spectrum when *p*-hydroxybenzoate binds to *p*-hydroxybenzoate hydroxylase. The changes are in the direction of strengthening the hydrogen bonds to the flavin. Equally significant is that bands at 1163, 1184, 1241, 1258, 1311, 1468, 1547, and 1595 cm^{-1} are not shifted, despite the indication from isotopic substitution studies of the flavin[13] that these bands are sensitive to changes in atoms involved in hydrogen bonding: N(1), N3), C(2)=O, and C(4)=O. Thus, introduction of *p*-hydroxybenzoate into the active site of *p*-hydroxybenzoate hydroxylase appears to cause a minimal perturbation of the flavin environment.

Binding 2,4-dihydroxybenzoate to the enzyme also causes a limited number of shifts in the band positions. In addition to those bands moved by *p*-hydroxybenzoate, 2,4-dihydroxybenzoate causes shifts in the 1241-, 1574-, and 1642-cm^{-1} bands. These additional shifts undoubtedly reflect the influence of the extra hydroxyl group on the active site environment.

In comparison to *p*-hydroxybenzoate and 2,4-dihydroxybenzoate, binding of any of the other ligands to the enzyme caused more changes to the Raman spectrum. Shifts of most bands were observed for the ternary complexes (enzyme + azide + *p*-hydroxybenzoate and enzyme + azide + 2,4-dihydroxybenzoate), for the effector (6-hydroxynicotinate), for *p*-aminobenzoate, and for the inhibitors (azide and chloride). These results indicate a major reorganization of the active site environment upon formation of these complexes. Again, the changes all point to stronger hydrogen bonds to the flavin. In the case of the ternary complexes and the 6-hydroxynicotinate, the hydrogen bond marker bands II and X are shifted at least 10 cm^{-1} in the direction predicted for stronger hydrogen bonding.[16]

Additional indication of conformation change upon ligand binding comes from a consideration of the relative intensities of the Raman bands. The apparent decrease in intensity of the bands in the 1450–1600-cm^{-1} region upon formation of the ternary and inhibitor complexes is indicative of the development of a ring-stacking interaction between the flavin and an active site aromatic amino acid residue. The modes between 1450 and 1600 cm^{-1} have been assigned primarily to rings II and III. This argues that the ring stacking involves rings II and III of the flavin. Several tyrosines have been identified in the active site, any one of which could stack with the flavin.

On the basis of the fluorescence polarization data, Steenis et al.[5] have also argued that *p*-hydroxybenzoate hydroxylase undergoes conformational changes in response to ligand binding. Such a condition has long been implicit in discussions on the "effector" role of substrates. A mechanism to account for the observation that reduction of the enzyme by NADPH required the presence of substrate (or effector) to proceed at a significant rate[2] seems to require an enzyme conformational change. This concept was recently strengthened by X-ray crystal studies which showed that the entrance to the substrate binding site becomes blocked after *p*-hydroxybenzoate is bound.[34] Our data are fully consistent with these findings.

The sensitivity of Raman spectroscopy allows us to readily discriminate between the effects of different ligands. It is interesting to note that the native substrate (*p*-hydroxybenzoate) appears to induce the least disruption in the active site when it binds to the enzyme. The alternate substrates 2,4-dihydroxybenzoate, *p*-aminobenzoate, and the effector 6-hydroxynicotinate cause progressively more structural disruption to the enzyme. This would be expected as the enzyme attempts to cope with functional groups for which it was not designed. Binding of the inhibitors, chloride and azide presents a different situation. These ligands do not bind in the substrate binding site, rather they are competitive with NADPH and appear to bind in the NADPH binding site.[2,4,5]

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Registry No. FAD, 146-14-5; *p*-hydroxybenzoate hydroxylase, 9059-23-8; *p*-hydroxybenzoate, 99-96-7; 2,4-dihydroxybenzoate, 89-86-1; *p*-aminobenzoate, 150-13-0; azide, 14343-69-2; chloride, 16887-00-6; 6-hydroxynicotinate, 5006-66-6.