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ARTICLE

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Analysis of the structure of the flavin-binding sites of flavocytochrome P450 BM3 using surface enhanced resonance Raman scattering

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Abstract Flavocytochrome P450 BM3, an FMN-deficient mutant (G570D), the component reductase and an FAD-containing domain were studied using surface enhanced resonance Raman scattering (SERRS). They were compared to spectra obtained from the free flavins FAD and FMN. For the holoenzyme and reductase domain, FMN is displaced during SERRS analysis. However, studies with the G570D mutant indicate that FAD is retained in its active site. Analysis of SERRS frequencies and intensities provides information on the nature of the flavin binding site and the planarity of the ring, and enables an interpretation of the hydrogen bonding environment around ring III of the isoalloxazine moiety. Hydrogen bonding is strong at N_3–H, C_2=O and C_4=O, but weak at N_5. Structural alteration of the FAD domain of P450 BM3 is caused by removal of the FMN-binding domain. Further, the hydrogen bond at N_3–H is lost and that at C_2=O is weakened and the isoalloxazine ring system in the FAD domain appears to adopt a more planar arrangement. Alterations in the environment of the FAD in its isolated domain are likely to relate to changes in the redox properties and suggest a close structural interplay of FAD with the FMN-binding domain in intact flavocytochrome P450 BM3.

Key words Raman · Surface enhanced resonance Raman scattering spectroscopy · Flavins · Flavocytochrome P450 BM3

Introduction

Flavins [usually flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN)] are vital cofactors in numerous enzyme systems. Flavin-containing proteins perform a myriad of functions in all organisms, including roles in the mammalian immune system (the microbicidal NADPH oxidase; Wallach and Segal 1996), drug detoxification (cytochrome P450 reductase; Strobel et al. 1989) and in the microbial synthesis of aromatic amino acids (chorismate synthase; Bornemann et al. 1995). The isoalloxazine ring of flavoproteins (Fig. 1) plays an essential role in the electron transfer processes catalysed by most flavoproteins (Morris and Bienstock 1986). Several spectroscopic techniques have been applied to studying the molecular properties of these proteins in order to understand more fully their functional basis as efficient electron donors/acceptors. Ultraviolet-visible absorption (Harbury et al. 1959), fluorescence (Platenkamp et al. 1980), infrared absorption (Holt and Cotton 1989), resonance Raman scattering (RR) (Müller et al. 1969) and surface enhanced resonance Raman scattering (SERRS) (Benecky et al. 1979) spectroscopies have all been employed to probe the intrinsic electronic and vibrational properties of flavins within many systems.

A flavoprotein class of particular interest are the NADPH-cytochrome P450 reductases (CPRs), which contain two flavins (one FAD and one FMN) and transfer electrons from NAD(P)H to the class II cytochromes P450 (P450s). The superfamily of P450 haemoproteins catalyse the monooxygenation of an enormous number of different substrate molecules and are found throughout nature. With one well-characterised exception [flavocytochrome P450 BM3 from *Bacillus megaterium* (Miles et al. 1992)], both

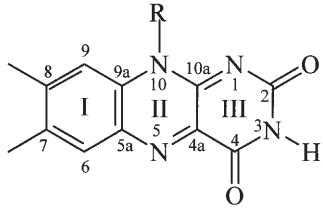

Fig. 1 The chemical structure of the isoalloxazine motif of flavins. The conventional ring numbering system used throughout associated literature (see Morris and Bienstock 1986) is noted in the diagram

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the class II P450s and the CPRs with which they interact are eukaryotic and membrane-bound. The membranous nature of these flavoenzymes has proved problematical as regards their overexpression, purification and spectroscopic characterisation. Despite a large pool of biochemical data within the literature, relatively little structural information has been collected about the flavin active sites of these proteins, an intrinsic factor in mediating the redox chemistry. However, the recent solution of the atomic structure of a trypsin-treated rat liver CPR has been a major breakthrough (Wang et al. 1997). This structure supports the hypothesis, based on primary sequence comparisons (Porter 1991), that CPRs are composed of two major domains: an $NADP^+$-ferredoxin reductase-like domain [containing FAD and responsible for removal of a pair of electrons (as a hydride ion) from NAD(P)H] and a flavodoxin-like domain (containing FMN and responsible for shuttling electrons from the FAD to the P450 haem). The atomic structure also indicates that the isoalloxazine rings of both flavins are closely spaced without intervening amino acid sidechains (Wang et al. 1997).

In flavocytochrome P450 BM3, the soluble fatty acid hydroxylase P450 is linked to its soluble P450 reductase in a single polypeptide (Narhi and Fulco 1986). The facts that both the P450 and reductase "domains" of the enzyme show strong amino acid similarity to their eukaryotic counterparts, that the atomic structure of the P450 domain has been solved and that intact P450 BM3 and its domains can be overexpressed to high levels and purified to homogeneity make P450 BM3 a highly attractive model system for spectroscopic studies.

Pre-resonance SERRS is used in this study to examine the nature of the flavin sites in flavocytochrome P450 BM3 and in its independently expressed reductase and FAD domains (Miles et al. 1992; Govindaraj and Poulos 1997). In addition, an FMN-free site-directed mutant of the flavocytochrome (G570D) (Klein and Fulco 1993) is examined. While resonance Raman is a good technique for the structural characterisation of chromophores such as haems, the concentration range suitable for study is limited and a strong fluorescence background reduces its utility for the study of many flavoproteins. The latter difficulty is particularly true when a weakly bound flavin is released into solution or is partially exposed. This represents a particular problem with P450 BM3, owing to the weak binding of FMN to this protein (Munro et al. 1996). SERRS has significant advantages over RR in providing very high quality vibrational data from flavins at low protein concentrations and in quenching fluorescence upon adsorption to a colloid surface so that the nature of the flavin can be established.

In this paper, forms of P450 BM3 with the flavin intact in the protein are established, evidence of distortion from planarity of FAD within its native site is provided and an altered H-bonding network around this moiety caused by the removal of the FMN domain is reported. Implications for electrochemical oxidation/reduction potentials and electron transfer are discussed.

Materials and methods

Colloid preparation

The preparation of the Lee and Meisel citrate-reduced silver colloid is described in a previous publication (Macdonald and Smith 1996). All pH readings were made using a Mettler 320 pH meter.

Enzyme preparation and storage

The method for expression and purification of intact flavocytochrome P450 BM3 has been presented in a previous publication (Miles et al. 1992). The FMN-deficient mutant holoenzyme G570D (Klein and Fulco 1993), the reductase domain (Miles et al. 1992) and the FAD domain (Govindaraj and Poulos 1997) of wild-type P450 BM3 were all purified by the same method as that used for the holoenzyme. Purified enzymes were concentrated by ultrafiltration to a volume of less than 2 ml and were dialysed at 4 °C into a 2000 × volume of storage buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM β-mercaptoethanol, 50% v/v glycerol]. Pure enzyme preparations were stored at −70 °C until use. No evidence for protein degradation (SDS-PAGE) or inactivation (loss of ferricyanide reductase activity or haem P420 formation by absorption spectroscopy) was observed for samples on thawing. Final concentrations of protein stocks were typically between 200 μM and 1 mM.

Materials

All chemicals used were of the highest purity available (>99%) and were purchased from Sigma, Fisons or Aldrich.

Sample preparations

Flavocytochrome P450 BM3 holoenzyme

A stock solution of 812 μM was diluted to 40.6 μM by the addition of 100 mM sodium phosphate (Fisons, Analar grade) buffer, pH 7.5 (buffer A). Five μl of this solution were added to 200 μl of 100 mM sodium phosphate buffer (pH 5.8), together with 15 μl of freshly prepared 1% w/v L-ascorbic acid (Sigma). This mixture was incubated on ice for 15 min to equilibrate and then added to 2 ml of cooled Ag colloid, immediately initiating aggregation of the colloidal suspension.

P450 BM3 reductase domain

A 6.15 μM protein solution was prepared by dilution of stock sample (615 μM) by the addition of buffer A. Fifteen μl of

this solution was incubated on ice with 75 µl of 100 mM phosphate buffer (pH 5.8) and 40 µl of freshly prepared 1% w/v L-ascorbic acid for 15 min. This solution was added to 400 µl of precooled Ag colloid, initiating aggregation of the colloidal suspension.

FMN-deficient P450 BM3 mutant G570D

A 42 µM solution of G570D was prepared by addition of buffer A to the 420 µM stock solution. Fifteen µl of this solution was added to 90 µl of 100 mM sodium phosphate buffer (pH 5.8) and 5 µl of freshly prepared 1% w/v L-ascorbic acid. The solution was incubated on ice for 15 min and added to 300 µl of cooled Ag colloid. This promoted aggregation, and the suspension was left to equilibrate for 10 min.

FAD domain

A solution of 6.94 µM was prepared from a stock (347 µM) in buffer A. Seventy five µl of 100 mM phosphate buffer (pH 5.5), 25 µl of freshly prepared 1% w/v L-ascorbic acid and 15 µl of protein solution were incubated on ice for 15 min. This mixture was added to a sample of 400 µl cooled Ag colloid, pre-acidified with 15 µl of 1% w/v L-ascorbic acid.

FMN and FAD

Stock solutions of FMN (5.11 mg/ml) and FAD (8.13 mg/ml) were prepared. Two µl of these stock solutions were made up to 1000 µl with distilled water. Ten µl of each solution was added to 1 ml of Ag colloid, followed by addition of 10 µl of 10% orthophosphoric acid, initiating aggregation. The colloidal suspensions were allowed to equilibrate for 15 min.

Raman instrumentation and data collection

SERRS spectra of cytochrome P450 BM3 holoenzyme were recorded from a 1 cm pathlength quartz fluorimeter cell using 457.9 nm and 514.5 nm excitation on an Anaspec-modified Cary 81 system with photon counting detection as described previously (Macdonald and Smith 1996). An Argon ion laser (Spectra Physics 2020/2045) was used to provide excitation. All other spectra were recorded from microtitre plate wells with a Renishaw Ramascope 2000, with excitation from a 25 mW 514.5 nm laser (Omnichrome). This system uses a CCD detector and collects backscattered light through an Olympus BH2-UMA microscope. SERRS spectra for the P450 BM3 holoenzyme recorded on the Cary instrument were collected 30 min after aggregation and all SERRS spectra recorded on the Renishaw system were collected after 10 min. This time difference was chosen to take account of the larger sample volumes used with the Cary system (ca. 2.2 ml) compared to the Renishaw system (400 µl) and ensure thermal equilibration in each case. In tests of sample stability, no significant time-dependent changes in the SERRS spectra of any of the proteins analysed were observed when measurements were made up to 4 h after sample aggregation, or after prolonged periods of sample irradiation.

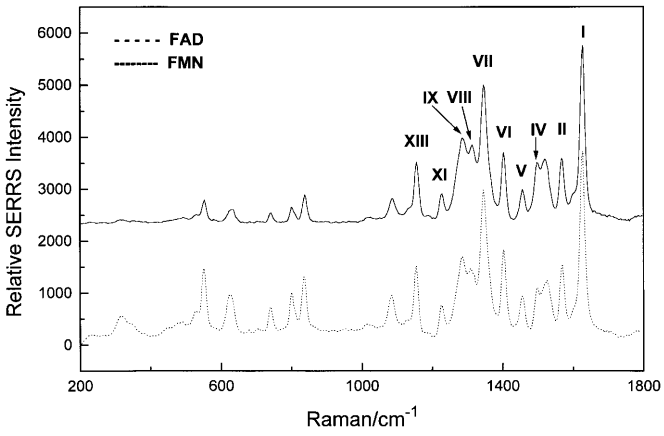

Fig. 2 SERRS of free flavins [FAD (*dotted line*) and FMN (*solid line*)] with 514.5 nm excitation. Flavin samples (FAD = 1.92×10^{-7} M; FMN = 2.09×10^{-7} M) were prepared as described in Materials and methods. Data acquisition was as described in the caption to Table 1. Positions of assigned bands (Table 1) are indicated on the figure. The wavenumbers of these bands (in units of cm^{-1}) are as follows for FMN (FAD): *I* 1629 (1629), *II* 1571 (1571), *IV* 1501 (1501), *V* 1459 (1459), *VI* 1405 (1405), *VII* 1350 (1350), *VIII* 1314 (1313), *IX* 1288 (1288), *XI* 1182 (1183), *XIII* 1158 (1159)

Results

FAD and FMN

SERRS of FMN and FAD with 514.5 nm excitation are presented in Fig. 2. Oxidised flavins and flavoproteins absorb at approximately 445–460 nm (Morris and Bienstock 1986). Using 514.5 nm excitation (some 55–70 nm from the flavin absorption peak), the scattering is pre-resonant rather than resonant, reducing the overall enhancement. This also affects relative intensities but has the advantage that electromagnetic surface selection rules can be employed in an approximate manner (Weitz et al. 1986; Creighton 1988; Otto et al. 1992), giving more information on the interaction with the surface. Comparison of SERRS frequencies for flavins with solution state vibrational frequencies (Table 1) indicate that the SERRS band positions are similar in many cases and correlate well with previously reported RR of flavins. The vibrational nomenclature for flavin bands is adopted from Bowman and Spiro (1981). Band positions are mostly 1–3 cm^{-1} lower than the cited literature study in Table 1, but these variations are within the range of published Raman values for flavins from other studies (Morris and Bienstock 1986). A poly-

Table 1 Vibrational frequencies from RR and SERRS studies of FMN and FAD

Band assignment[a]	RR of FMN (cm^{-1}) pH 8.5[b]	RR of FAD (cm^{-1}) pH 8.5[b]	SERRS of FMN (cm^{-1})[c]	SERRS of FAD (cm^{-1})[c]
I	1628	1629	1629	1629
II	1581	1582	1571	1571
IV	1501	–	1501	1501
V	1462	1461	1459	1459
VI	1407	1408	1405	1405
VII	1353	1353	1350	1350
VIII	–	–	1314	1313
IX	1278	1280	1288	1288
X	1256	1255	–	–
XI	1228	1228	1230	1231
XII	1183	1183	1182	1183
XIII	1158	1160	1158	1159
–	–	–	1088	1090
–	–	–	839	841
–	–	–	805	805
–	–	–	744	745
–	–	–	631	636
–	–	–	555	555

[a] Nomenclature adopted from Abe and Kyogoku (1987) and Bowman and Spiro (1981)
[b] See Dutta et al. (1978)
[c] Results from this study with 514.5 nm excitation (25 mW), using 1 scan of 5 s duration. Sample concentrations: 1.92×10^{-7} M (FMN) and 2.09×10^{-7} M (FAD)

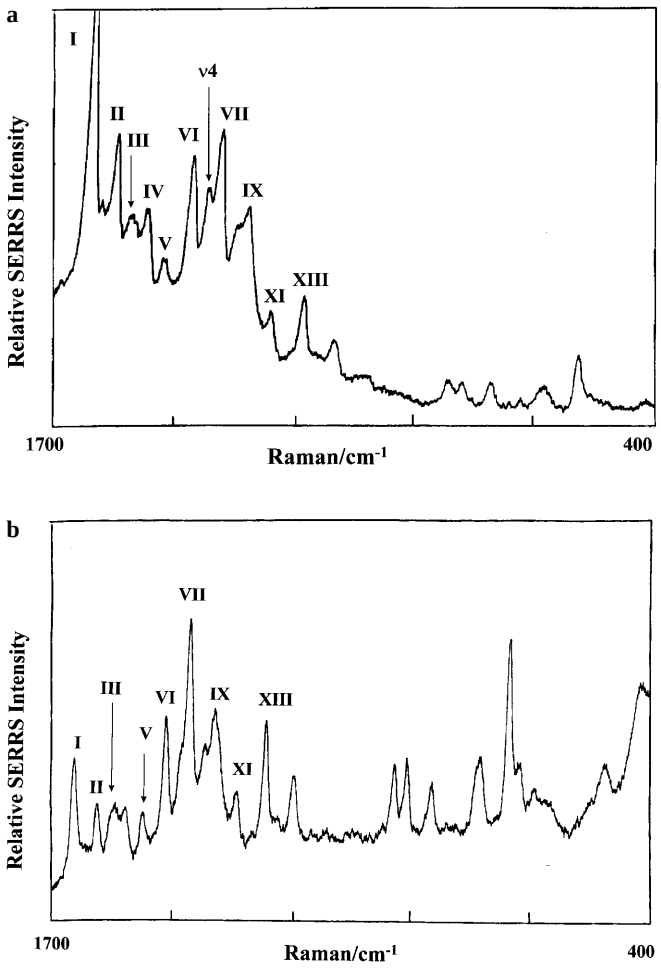

Fig. 3 SERRS of flavocytochrome P450 BM3 holoenzyme (9.14×10^{-8} M) with excitation at 457.9 nm (**a**) and 514.5 nm (**b**). Samples were prepared as detailed in Materials and methods. Data were acquired as described the caption to Table 2. Positions of assigned bands (Table 2) are indicated on the figures. The wavenumbers of these bands (in units of cm^{-1}) are as follows: **a** *I* 1630, *II* 1575, *III* 1531, *IV* 1503, *V* 1461, *VI* 1406, *v4* 1372, *VII* 1347, *IX* 1284, *XI* 1230, *XIII* 1159; **b** *I* 1629, *II* 1574, *III* 1530, *IV* 1503, *V* 1460, *VI* 1406, *v4* (shoulder) 1372, *VII* 1347, *IX* 1282, *XI* 1229, *XIII* 1157

meric citrate layer coats the colloid surface in Lee and Meisel colloid (Munro et al. 1995), producing a negative surface to which the flavins will adsorb. It should be noted that the larger frequency variations for flavin bands II and XI (ring III motions) are associated with interactions with the citrate surface, as discussed later.

To examine the possibility that, rather than adsorbed flavin, an Ag/flavin complex is formed on the colloidal surface, such a complex was prepared. The SERRS (data not shown) gave a strong broad band at 1322 cm^{-1}. Broad features were also apparent in the 1500–1600 cm^{-1} region and bands in the 1200–1300 cm^{-1} region were of low intensity. None of these spectral characteristics were apparent in the SERRS spectra of FAD or FMN, nor any of the flavin-containing proteins examined subsequently, indicating that no complex is formed under the conditions used in this study.

Flavocytochrome P450 BM3

The Soret band of the haem is located at ~418 nm with α- and β-bands at about 570 nm and 535 nm, respectively. Flavin absorbance maxima are at 385 nm and 456 nm and appear as shoulders on the Soret band. SERRS of P450 BM3 was obtained with 514.5 nm and 457.9 nm excitation (Fig. 3) and the vibrational data are shown in Table 2. The flavin bands from P450 BM3 occur at frequencies consistent with previous studies of SERRS of FMN/FAD and solution state RR of flavins. This and the SERRS of the silver complex eliminate the possibility of formation of silver-flavin complexes. An additional band appears at 1372 cm^{-1}, which has been assigned to ν_4 from the haem chromophore and is quite strong with 457.9 nm excitation (Table 2). ν_4 is the most intense haem band for SERRS and RR studies at 457.9 nm and 514.5 nm for other P450s (Rospendowski et al. 1991; Miles et al. 1992). The fact that only the strongest haem band (ν_4) is observed in this study, and even this band is only seen very weakly with 514.5 nm excitation, indicates that other haem bands do not contribute significantly to the flavin-dominated SERRS spectra of P450 BM3. Vibrational frequencies for modes sensitive to bonding interactions (bands II, VI, IX and XI) are different from those of adsorbed FMN/FAD, indicating an altered local environment for the flavins.

Table 2 SERRS of flavocytochrome P450 BM3 excited with 457.9 nm (100 mW) and 514.5 nm (50 mW) radiation. P450 BM3 concentration for both studies was 9.14×10^{-8} M. Each spectrum was obtained with 1 scan. Reductase domain concentration was 1.74×10^{-7} M and was excited with 514.5 nm radiation (25 mW), using 1 scan of 20 s duration. For the reductase domain, a weak signal from band X is located at 1253 cm^{-1}

Vibrational nomenclature[a]	CYP102 SERRS at 457.9 nm (cm^{-1})	CYP102 SERRS at 514.5 nm (cm^{-1})	SERRS of reductase domain at 514.5 nm (cm^{-1})
I	1630	1629	1629
II	1575	1574	1574
III	1531	1530	1531
IV	1503	1503	1502
V	1461	1460	1461
VI	1406	1406	1404
ν_4	1372	1372	–
VII	1347	1347	1347
VIII	1310	1309	–
IX	1284	1282	1281
XI	1230	1229	1229
XII	–	–	–
XIII	1159	1157	1158
–	1087	1083	1085
–	836	838	
–	804	806	
–	745	743	
–	627	623	
–	556	552	552

[a] see Abe and Kyogoku (1987) and Bowman and Spiro (1981)

Reductase domain

The SERRS spectrum was recorded at 514.5 nm excitation (Table 2, Fig. 4). The vibrational frequencies of the flavin modes and the relative intensities are very similar to those from P450 BM3.

Mutant G570D

The SERRS spectrum of FMN-deficient P450 BM3 mutant G570D was recorded at 514.5 nm. Vibrational frequencies and assignments are presented in Table 3 together with RR values from other flavoproteins. The most intense bands in the spectrum are VI (1403 cm^{-1}) and VII (1347 cm^{-1}). The results for FMN-deficient protein exhibit the vibrational motifs of flavins uncomplexed with metal ions. Altered frequencies (comparison of bands II, IX and XI in Tables 1 and 2) indicate a distinct environment for FAD within G570D. Significant relative intensity differences from solution state RR flavin, SERRS of free FMN/FAD and SERRS of wild-type P450 BM3 including the FMN and FAD domain indicate that FAD is retained within the protein, as discussed later.

FAD domain

SERRS of the FAD domain of P450 BM3 recorded with 514.5 nm excitation (Table 3, Fig. 4) gives certain flavin

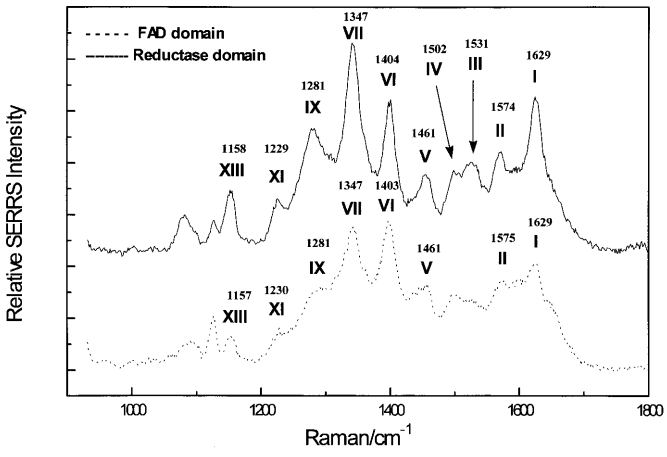

Fig. 4 SERRS of the reductase (1.74×10^{-7} M) and FAD (1.96×10^{-7} M) domains of flavocytochrome P450 BM3 with excitation at 514.5 nm. Samples were prepared as described in Materials and methods. Data were acquired as stated in the caption to Tables 2 and 3. Positions of assigned bands are indicated in Tables 2 and 3

Table 3 SERRS and RR band frequencies and assignments of flavoproteins

Vibrational assignment[a]	SERRS of G570D mutant (cm^{-1})[b]	RR of GR (cm^{-1})[c]	RR of MCAD (cm^{-1})[d]	SERRS of FAD domain (cm^{-1})[e]
$\nu C_2=0$	1664	–	–	1667
I	1632	1627	1624	1628
II	1572	1580	1583	1575
V	1460	–	–	1461
VI	1403	1407	1407	1403
VII	1347	1353	1352	1347
IX	1284	1281	–	1281
X	–	1246	1254	–
XI	1231	1224	1231	1230
XII	–	1180	1182	–
XIII	1161	–	1162	1157

[a] See Abe and Kyogoku (1987) and Bowman and Spiro (1981)
[b] Results from the present study for the FMN-deficient mutant of P450 BM3, G570D, recorded with 514.5 nm excitation (25 mW), using 10 scans each of 60 s duration. Protein concentration is 1.55×10^{-5} M
[c] Glutathione reductase (GR)
[d] Medium chain fatty acyl coA-dehydrogenase (MCAD). GR and MCAD RR values obtained from Strobel et al. (1996)
[e] Results from the present study for the FAD-containing domain from P450 BM3. Sample concentration was 1.96×10^{-7} M and the sample was excited with 514.5 nm radiation (25 mW) using 1 scan of 30 s duration. For mutant G570D and the FAD domain, weak signals from band X are located at 1256 cm^{-1} and 1246 cm^{-1}, respectively

frequencies which are similar to those for G570D [i.e. bands VI (1403 cm^{-1}) and XI (1230 cm^{-1})]. However, other modes give frequencies significantly different from those observed previously. These include bands II (1575 cm^{-1}) and IX (1281 cm^{-1}) and are representative of a distinct local environment around the flavin.

In agreement with these findings, the visible region CD spectrum of the FAD domain shows positive ellipticity over

the region from 320 nm to 400 nm, whereas the reductase has negative ellipticity over the entire range from 300 nm to 500 nm. This suggests that the local environment of the FAD is altered significantly in the absence of the FMN-containing domain.

Discussion

SERRS of free FMN and FAD

Theoretical predictions of flavin vibrational frequencies from three independent research groups have been compared with isotopic labelling studies (Kitagawa et al. 1979; Bowman and Spiro 1981; Spiro et al. 1982; Abe and Kyogoku 1987; Lively and McFarland 1990) and, in general, consensus has been reached on the nature of inplane flavin. Lively and McFarland (1990) incorporated H-bonding effects into their theoretical program with good agreement between experimental and theoretically predicted Raman frequencies for flavin species. Qualitative application of their interpretations to our studies can provide information on the H-bonding environment experienced by each flavin species within its native site.

Hydrogen bonding environment, isoalloxazine distortion and surface orientation

To provide a basis for our interpretations, SERRS studies were performed on the free flavins FMN and FAD. Bands II and III have significant atomic displacements from ring III of the isoalloxazine moiety. Band II has a large mixing of the five bonds symmetrically positioned on the $C_{4a}C_{10a}$ plane (see Fig. 1 for atomic numbering). Band III is assigned to a mode from the diazabuta-1,3-diene group comprising $\nu(C_{4a}N_5)$, $\nu(C_{4a}N_{10a})$ and $\nu(C_{4a}N_1)$. The low frequencies (relative to RR studies) for bands II and VI for adsorbed flavins, coupled with the high frequency for band XI, are consistent with an electrostatic/H-bonding pattern which is strong at $C_4{=}O$, $C_2{=}O$ and N_1, and weak at N_5. A strong H-bond at position $N_3{-}H$ is inferred by the high frequencies for bands IX–XI. The weak nature of the H-bonding environment around N_5 is supported by the low frequency for band III (1525 cm^{-1}). This is in contrast to the strong H-bonding at this position for solution state flavins (band III at 1547 cm^{-1}).

Despite the large body of flavin RR/SERRS data in the literature, little attention has been directed towards the possibility of distortion arising in this non-aromatic isoalloxazine system. Recently, Tegoni et al. (1997) published an article which addressed this point. They discovered a strict relationship between the frequencies of bands II and III for flavoproteins when the isoalloxazine ring is planar. Deviations arose when distortion from planarity occurred. Two distinct types of out-of-plane distortions were identified. One is a "butterfly" bending along the virtual $N_5{-}N_{10}$ axis, the other is a "propellor" twisting around the long axis which passes through the three rings of the isoalloxazine chromophore. This long axis is perpendicular to the virtual $N_5{-}N_{10}$ axis. Neither the form nor the extent of structural deformation can be predicted at present. The SERRS frequencies from free FAD and FMN deviate from the band II/III relationship. This suggests that the adsorbed flavins are structurally distorted.

Supporting evidence for this distortion arises from analysis of the relative SERRS intensities, which can provide some information on the orientation of the plane of the prosthetic group of the chromophore relative to the silver surface (Macdonald and Smith 1996). SERRS intensities from adsorbed FMN and FAD are very similar but markedly different from the equivalent RR values. The application of electromagnetic selection rules (Weitz et al. 1986; Creighton 1988; Otto et al. 1992) can be used to indicate the surface ordering of the flavin chromophores. In the simplest expression of these rules and ignoring symmetry, bands from adsorbed molecular species with displacements which coincide with the radial field direction from small colloidal metal particles will be enhanced with respect to bands with tangential displacements (Weitz et al. 1986). Thus, the approximate orientation of the chromophore with respect to the surface can be obtained by comparison of SERRS with RR data.

Compared with RR, there is a marked increase in the relative intensity of vibrations I, III and XII, and a decrease in bands II, VII, X and XIII, in SERRS. It appears that flavin bands with significant ring I displacements are selectively enhanced, suggesting that this ring is positioned at a more vertical position than rings II or III. The absence of band X suggests that surface adsorption is via ring III, indicating H-bonding interference in the vibrational motion of the internal peptide linkage ($HN_3{-}C_4O$) in ring III. The flatter arrangement for rings II and III probably arises from favourable H-bonding interactions between the polarised groups (which reside mostly on ring III) and the colloidal surface, mediated by the aqueous environment. The acidic nature of the colloidal suspension would aid these interactions.

Thus, strong H-bonding character at atomic positions N_1 and $C_4{=}O$ and an out-of-plane distortion of the isoalloxazine ring are indicated from the SERRS of adsorbed FMN/FAD. Weak H-bonding is indicated at the N_5 sites. Relative band intensities suggest adsorption of flavins via ring III, which adopts a relatively flat orientation on the surface.

Evidence for flavin displacement in P450 BM3 and its reductase domain

The SERRS spectra obtained from P450 BM3 are quite different in relative intensity to those of SERRS from free FMN/FAD. With 514.5 nm excitation, the SERRS intensities are similar to those expected for RR from a flavin. The strong SERRS from flavin modes rather than haem modes may suggest that protein adsorption occurs in a way which positions the flavin chromophores closer to the surface than the haem. The relationship between SERRS/RR

intensities strongly suggests that flavin is removed from its binding pocket and is randomly positioned either within the protein matrix or near its surface at the protein/colloid interface. The differences compared to FAD/FMN for bands II (+3 cm^{-1}), IX (−5 to −7 cm^{-1}) and XI (−1 cm^{-1}) and lower frequency modes (see Tables 1 and 2) indicate a weaker H-bonding environment at atomic positions $C_2=O$, N_3-H and $C_4=O$ than for adsorbed FMN/FAD. The high frequency of band IV suggests the electrostatic/H-bonding environment around position N_5 is stronger than for adsorbed flavin. Distortion of the plane of the rings is indicated by the observed frequency deviation from the strict band II/band III relationship which exists for planar isoalloxazine ring systems. All this evidence supports the model of randomly distributed flavin displaced from its binding pocket and adsorbed on the surface with the protein. The most likely alternative, randomly oriented protein, is not consistent with the pattern of changes, particularly since chromophores held away from the surface would give weaker signals.

The qualitative similarity of the SERRS frequencies and relative intensities between P450 BM3 and its reductase domain, and the high signal-to-noise ratio, indicate the presence of displaced flavin for adsorbed reductase domain. The H-bonding characteristics suggested to occur in P450 BM3 are also evident. SERRS signals from glucose oxidase have previously been assigned to displaced flavin and in that case the repurified enzyme exhibited no SERRS activity (Lee et al. 1987).

Evidence supporting native-like FAD retention

To investigate whether one or both flavins are displaced upon adsorption on the surface, the P450 BM3 mutant G570D was studied. This point mutation prevents incorporation of FMN (Klein and Fulco 1993). G570D does contain FAD and retains high ferricyanide reductase activity. SERRS indicates that FAD is retained within its active site upon surface adsorption. The SERRS intensities provide a unique pattern which differs from the previous cases. The more intense modes have a large contribution from ring II of the isoalloxazine moiety. This is not observed for SERRS studies of adsorbed FAD/FMN, P450 BM3 or reductase domain and indicates a unique alignment of flavin in this mutant. A distinct difference in spectral quality is observed for G570D compared to all the other systems discussed previously. The low signal-to-noise ratio observed despite using concentrations up to >100 times higher than with the previous systems suggests that the chromophore is spaced off the surface and is consistent with retention in the pocket. Further, adsorbed G570D can be irradiated for prolonged periods of time with no increase in signal strength or change in relative intensities. Both Lee et al. (1987) and Holt and Cotton (1987) provided evidence that prolonged irradiation photodecomposes glucose oxidase. They reported signal intensity increases with longer illumination times, which were attributed to photoinduced decomposition releasing FAD from glucose oxidase.

H-bonding characteristics for mutant G570D

The frequency of band II (1572 cm^{-1}), coupled with the low frequency for band VI, indicate a stronger electrostatic/H-bonding environment around $C_2=O$ relative to the other cases discussed. This is supported by the observation of a band, assigned to a $C_2=O$ stretch, at 1664 cm^{-1}. Strong H-bonding to adsorbed and solution state flavins is indicated for N_3-H based upon the high frequencies for bands X and XI. The fact that band X is weak is likely as a consequence of limited vibrational capacity – due directly to the H-bonding properties of the flavin. H-bonding is indicated round $C_4=O$, but it is weaker than that observed for adsorbed FAD and FMN. H-bonding at N_5 is weak. By applying another of Tegoni's relationships (Tegoni et al. 1997), the number of H-bonds (≤3.4 Å) expected at atomic positions N_1, O_2, N_3 and O_4 may be calculated from the increase in frequency of band X. The frequency for band X indicates that there are six H-bonds associated with positions N_1, O_2, N_3 and O_4 for the FAD moiety of G570D.

Domain-domain structural effects

To probe the environment surrounding the FAD-binding site, the FAD/NADPH domain of P450 BM3 was purified and characterised using SERRS. In the only atomic structure of a P450 reductase (Wang et al. 1997), the FAD and FMN are positioned within facile distance for electron transfer. Although the relative positioning of the flavins groups in P450 BM3 may be slightly different from the microsomal reductase, it would intuitively be expected that removal of the FMN domain would significantly alter structural properties of the FAD-binding site. Visible region CD studies on the FAD domain indicate an altered environment relative to the reductase and holoenzyme. SERRS data suggest a distinct state for the isoalloxazine ring in the FAD domain. These localised structural effects do not destabilise the protein, as experimental evidence from physical and chemical treatments of the FAD domain indicate comparable flavin stability with P450 BM3 and the reductase domain (A. W. Munro, unpublished data).

Hydrogen bonding characteristics of FAD domain

SERRS indicates a distinctive electrostatic/H-bonding pattern for the FAD domain. The low frequency of band VI at 1403 cm^{-1} suggests stronger electrostatic/H-bonding at position $C_2=O$ than for solution state or adsorbed FMN/FAD. However, it is weaker than for G570D, as reflected in the $C_2=O$ upshift to 1667 cm^{-1}. A weaker electrostatic environment surrounds $C_4=O$. This is suggested by band II's intermediate frequency. A weak bonding environment at N_3-H is suggested by the low frequency of band X. Applying Tegoni's observation on H-bonding character (Tegoni et al. 1997), a total number of five H-bonds overall at positions N_1, O_2, N_3 and O_4 is predicted, but the distribution has changed compared to G570D. For example, it ap-

pears that the N_3–H H-bond is lost upon removal of the FMN-binding domain.

The SERRS results for the FAD domain, however, do show some similarities to G570D. The relative intensities of bands VI/VII, II/VI and II/VII are comparable to G570D. This suggests that the flavin in the FAD domain adopts a similar position to that in G570D. A higher signal-to-noise ratio is observed for FAD domain but the results are inconsistent with protein-displaced flavin. There are few similarities between the SERRS intensities and frequencies of P450 BM3 and the FAD domain. The data also bear little resemblance to SERRS from free FAD/FMN. The results suggest that the flavin is positioned close to the surface, but retained within the protein.

Catalytic relevance of domain-domain interactions

The redox couples for the individual domains of P450 BM3 have been determined by electrochemical titrations (Daff et al. 1997). For the FAD domain, these couples were +23 mV and +35 mV, respectively, relative to the reductase domain. These observations can be explained by changes in the polarity and/or electrostatics in the immediate protein-flavin environment and by an alteration of the three-dimensional structure of the isoalloxazine ring. The SERRS studies indicate that removal of the FMN-binding domain significantly alters the H-bonding characteristics around the FAD active site. In addition to altered electrostatics within the pocket, analysis of the band II/band III SERRS frequencies suggests that the isoalloxazine ring has become planar in the absence of the FMN domain. Further evidence for an altered geometric arrangement for the FAD domain are the significantly downshifted band I frequency at ~1628 cm^{-1}, the loss of a H-bond at N_3–H and the 3 cm^{-1} upshift of the C_2=O vibration.

A similar effect was observed on pyruvate binding to flavocytochrome b_2, where there was an increase of 80 mV for the oxidised/semiquinone couple (Tegoni et al. 1997). No flavin distortion was observed in this case and the effect was largely attributed to a weakened H-bonding environment around the C_4=O position on ring III of the FAD. The SERRS data suggest that, on removal of the FMN-binding domain, a weaker electrostatic/H-bonding regime arises at C_2=O and, to a lesser extent, at C_4=O. The N_3–H bond appears to have lost its H-bond. Thus, in the two cases cited it appears that the electrochemical redox couple is very sensitive to electrostatic effects on the C_2=O, N_3–H and C_4=O structural hinge. This is important when one considers binding of the physiological reductant, NADPH.

Although polarity and structural effects induced by the removal of the FMN-binding domain have been observed, the relative positioning of the two flavins is critical for efficient and controlled delivery of electrons from NADPH for oxygen activation and stereo-specific oxidation of fatty acids. FMN-deficient mutant G570D does not function as a fatty acid oxidase, indicating that the formation of a ternary structure involving precise positioning of the FAD, FMN and NADPH reductant is required for catalysis. The easy removal of FMN from the intact P450 BM3 enzyme precludes the present form of SERRS being used to elucidate that system and technique improvements are required.

Conclusions

By carefully controlling experimental conditions, SERRS can be obtained from flavins retained within the native protein binding pocket. The technique can provide information on the chemical environment around the flavin and qualitative information on the angle of the flavin ring system to the silver surface. This can be helpful in establishing the nature of protein/surface contacts. Nanomolar protein concentrations can be used and fluorescence is quenched, making the technique more widely applicable than resonance Raman scattering.

The unique SERRS profile of G570D shows that FAD is retained within its active site. FMN is the displaced flavin in the holoenzyme and reductase studies. These findings are consistent with previous studies of the relative strengths of binding of FAD and FMN to the enzyme (Munro et al. 1996). SERRS data from mutant G570D is consistent with a H-bonding environment which is strong at N_3–H (≤2.5 Å), C_2=O and C_4=O, but weak at N_5. Removal of the FMN-binding domain leads to structural alteration of the FAD moiety in the FAD domain. An apparent loss of the H-bond at N_3–H and a planar isoalloxazine ring system are features of the FAD domain. All other studies indicate that FAD chromophore is distorted. The alteration in the FAD structure induced by domain-domain interactions may explain alterations observed in the redox couples of the FAD between the reductase and the FAD domain (Daff et al. 1997) and may be important for efficient communication and control of electron transfer to the haem in the holoenzyme.

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References

Abe M, Kyoguku Y (1987) Vibrational analyses of flavin derivatives – normal coordinate treatments of lumiflavin. Spectrochim Acta Part A 43: 1027–1037

Benecky M, Li TY, Schmidt J, Frerman F, Watters KL, McFarland JT (1979) Resonance Raman study of flavins and the flavoprotein fatty acyl coenzyme A dehydrogenase. Biochemistry 18: 3471–3746

Bornemann S, Ramjee MK, Balasubramanian S, Abell C, Coggins JR (1995) *Escherichia coli* chorismate synthase catalyzes the conversion of (6*S*)-6-fluoro-5-enolpyruvylshikimate-3-phosphate to 6-fluorochorismate – implications for the enzyme mechanism and the anti-microbial action of (6*S*)-6-fluoroshikimate. J Biol Chem 270: 22811–22815

Bowman WD, Spiro TG (1981) Normal mode analysis of lumiflavin and interpretation of resonance Raman spectra of flavoproteins. Biochemistry 20: 3313–3318

Creighton JA (1988) The selection rules for surface-enhanced Raman spectroscopy. In: Clark RJH, Hester RE (eds) Spectroscopy of surfaces. Wiley, New York, pp 37–95

Daff SN, Chapman SK, Turner KL, Holt RA, Govindaraj S, Poulos TL, Munro AW (1997) Redox control of the catalytic cycle of flavocytochrome P-450 BM3. Biochemistry 36: 13816–13823

Dutta PK, Nestor JR, Spiro TG (1978) Resonance coherent anti-Stokes Raman scattering (CARS) spectra of flavin adenine dinucleotide, riboflavin binding protein and glucose oxidase. Biochem Biophys Res Commun 83: 209–216

Govindaraj S, Poulos TL (1997) The domain architecture of cytochrome P450 BM3. J Biol Chem 272: 7915–7921

Harbury H, Lanoue KF, Loach P, Amick RM (1959) Molecular interactions of isoalloxazine derivatives II. Proc Natl Acad Sci USA 45: 1708–1717

Holt RE, Cotton TM (1987) Free flavin interference in surface enhanced resonance Raman spectroscopy of glucose oxidase. J Am Chem Soc 109: 1841–1845

Holt RE, Cotton TM (1989) Surface enhanced resonance Raman and electrochemical investigation of glucose oxidase catalysis at a silver electrode. J Am Chem Soc 111: 2815–2821

Kitagawa T, Nishima Y, Kyogoku T, Yamano N, Ohishi N, Takai-Sutuki A, Yagi K (1979) Resonance Raman spectra of carbon-13- and nitrogen-15-labelled riboflavin bound to egg-white flavoprotein. Biochemistry 18: 1805–1811

Klein ML, Fulco AJ (1993) Critical residues involved in FMN-binding and catalytic activity in cytochrome P-450 BM3. J Biol Chem 268: 7553–7561

Lee N-S, Hsieh Y-Z, Morris MD, Schopfer LM (1987) Re-interpretation of surface enhanced resonance Raman scattering of flavoproteins on silver colloids. J Am Chem Soc 109: 1358–1363

Lively CR, McFarland, JT (1990) Assignments and the effect of hydrogen bonding on the vibrational normal modes of flavins and flavoproteins. J Phys Cem 94: 3980–3994

Macdonald IDG, Smith WE (1996) Orientation of cytochrome *c* adsorbed on a citrate-reduced silver colloid surface. Langmuir 12: 706–713

Miles JS, Munro AW, Rospendowski BN, Smith WE, McKnight J Thomson AJ (1992) Domains of the catalytically self-sufficient cytochrome P-450 BM3: genetic construction, overexpression, purification and spectral characterization. Biochem J 288: 503–509

Morris MD, Bienstock RT (1986) Resonance Raman spectroscopy of flavins and flavoproteins. In: Clark RJH, Hester RE (eds) Spectroscopy of biological systems, Vol 1. Wiley, New York, pp 395–442

Müller F, Massey V, Heizmann C, Hemmerich P, Lhoste JM, Gould DC (1969) The reduction of flavins by borohydride: 3,4-dihydroflavin; structure, absorption and luminescence. Eur J Biochem 9: 392–401

Munro AW, Lindsay JG, Coggins JR, Kelly SM, Price NC (1996) Analysis of the structural stability of the multi-domain enzyme flavocytochrome P-450 BM3. Biochim Biophys Acta 1296: 127–137

Munro CH, Smith WE, Garner M, Clarkson J, White PC (1995) Characterization of the surface of a citrate-reduced colloid optimized for use as a substrate for surface-enhanced resonance Raman scattering. Langmuir 11: 3712–3720

Narhi LO, Fulco AJ (1986) Characterization of a catalytically self-sufficient 119,000 Dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium*. J. Biol Chem 261: 7160–7169

Otto A, Mrozek I, Grabhorn H, Akemann W (1992) Surface enhanced Raman scattering. J Phys Condens Matter 4: 1143–1212

Platenkamp RJ, Osnagrügge HD, Visser AJWG (1980) High resolution fluorescence and excitation spectroscopy of N_3-undecyllumiflavin in *n*-decane. Chem Phys Lett 72: 104–111

Porter TD (1991) An unusual, yet strongly conserved, flavoprotein reductase in bacteria and mammals. Trends Biochem Sci 16: 154–158

Rospendowski BN, Kelly K, Wolf CR, Smith WE (1991) Surface enhanced Raman scattering from cytochromes P-450 adsorbed on citrate-reduced silver sols. J Am Chem Soc 113: 1217–1225

Spiro TG, Bowman WD, Dutta PK, Benecky MJ (1982) Normal mode analysis of lumiflavin and the interpretation of flavoprotein resonance Raman spectra. In: Massey V, Williams CH (eds) Flavins and flavoproteins. Elsevier, Amsterdam, pp 554–557

Strobel HW, Nadler SG, Nelson DR (1989) Cytochrome P-450-cytochrome P-450 reductase interactions. Drug Metab Rev 20: 519–533

Strobel HW, Hodgson AV, Shen S (1996) NADPH cytochrome P450 reductase and its structural and functional domains. In: Ortiz de Montellano PR (ed) Cytochrome P450: structure, mechanism and biochemistry. Plenum Press, New York, pp 225–244

Tegoni M, Gervais M, Desbois A (1997) Resonance Raman study on the oxidized and anionic forms of flavocytochrome b_2 and L-lactate monooxygenase: influence of the structure and environment of the isoalloxazine ring on the flavin function. Biochemistry 36: 8932–8946

Wallach TM, Segal AW (1996) Stoichiometry of the subunits of flavocytochrome b_{558} of the NADPH oxidase of phagocytes. Biochem J 320: 33–38

Wang M, Roberts DL, Paschke R, Shea TM, Masters BSS, Kim J-J (1997) Three dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. Proc Natl Acad Sci USA 94: 8411–8416

Weitz DA, Moskovits M, Creighton JA (1986) Surface enhanced Raman spectroscopy with emphasis on liquid-sol interfaces. In: Hall RB, Ellis AR (eds) Chemistry and structure at interfaces – new laser and optical techniques. VCH, Weinheim, pp 197–243