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Observation of Structural Variation and Spin State Conversion of Cytochrome P450 CYP2B4 on Binding of Sterically Different Substrates

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Addition of sterically different substrates to CYP2B4 suggests perturbation of the environment of the haem ring and differing degrees of **6cls-5chs** conversion. Strain on the ring, by implication induced by changes in the surrounding protein environment, is demonstrated by the emergence of the ν_{29} band upon laurate binding. Furthermore, intensity differences and frequency shifts for non-Raman active (E_u) modes appear to be substrate dependent and are sensitive to haem/substrate interactions. The likelihood is that substrate induced distortion of the haem will have an effect on catalytic activities and attention needs to be directed towards substrate pocket interactions. **SERRS** (Surface enhanced resonance Raman scattering) provides a unique and sensitive spectroscopic probe of these interactions.

INTRODUCTION

The large number of cytochromes P450 characterised to date reflect the diversity of exogenous and endogenous compounds oxygenated via the cytochrome P450 ‘monooxygenase’ cycle [1-5]. This broadly based oxidation cycle has led to cytochromes P450 being implicated in the activation of known carcinogens such as nitrosamines and polycyclic aromatic hydrocarbons [6,7]. Thus, an understanding of P450/substrate interactions in situ is of importance to aid understanding of P450-dependent metabolism.

The active pocket contains a ‘b’ type haem which is coordinated axially by a cysteine thiolate (Cys-436) from the cytochrome P450 CYP2B4 apoprotein [8]. A weakly bound and easily exchangeable axial aquo species serves as the molecular oxygen binding site during catalysis [8]. Induced coordination changes and spin configuration alterations of the haem iron are intrinsically linked to enzymatic activity in the oxidation cycle.

Surface enhanced resonance Raman scattering (**SERRS**) has been successfully applied to haemoproteins [11-19], including cytochromes P450, providing information using nanomolar amounts of protein on the geometry, conformation and spin states of active enzymes adsorbed onto microscopically roughened metal (Ag) surfaces. This technique derives its sensitivity (~10000 times better than resonance Raman) from adsorption of the protein onto surfaces where amplification of the scattering is gained by coupling resonance Raman scattering with plasmons (electron waves) on the metal surface. Fluorescence quenching (problematic for resonance Raman) occurs at these surfaces extending further the range of proteins which can be studied. Using **SERRS** it has proved possible to detect active site differences with CYP2B4 in low concentration under buffer.

Interpretation of **SERRS** requires the use of the extensive resonance Raman studies of model compounds [20-22] and haem systems [23-26]. Clear assignments of the major in-plane

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vibrations have been formulated. These modes provide information on the oxidation and spin state, nature of axial ligation and geometrical strain on the porphyrin ring. Thus, the spectrum provides considerable information on the structural state of the haem pocket.

MATERIALS AND METHODS

Purification and preparation of phenolbarbital induced rabbit liver cytochrome P-450 CYP2B4 was performed according to methodology outlined by *Wolf et al* [27]. Colloidal silver was prepared using a modified [17] *Lee* and *Meisel* method. The prepared suspensions were stable for 6-8 weeks.

Cytochrome P-450 samples were supplied in 10mM phosphate buffer containing 20%w/w glycerol and 0.1mM of both ethylenediaminetetraacetic acid (Na_4 salt) and dithiothreitol. Protein concentrations varied from 15.2nmol/ml to 19.2nmol/ml in the aforementioned buffer. 10-20μl of cytochrome P-450 was incubated in ice in the presence of 100mM phosphate buffer (Fisons,Analar Grade) at a pH of 7.35. If required for aggregation, a freshly prepared solution of 1%w/v L-ascorbic acid (Sigma,>99%) was used.

The protein solution was added to 2ml of cooled silver colloid and incubated in ice. Insertion into a compartment with circulating coolant (iced water) maintained the sample temperature at 2-6°C throughout the Raman measurement process. Substrates dimethyldiaminoantipyrine [aminopyrine] (Aldrich,>97%) and sodium laurate (Sigma,>99%) were prepared as 11.46mM and 25mM methanolic solutions respectively. Preacidification with 20μl of ascorbic acid was followed by addition of 200μl of aminopyrine and 10μl ascorbic acid to the protein aggregated colloid. Sodium laurate (100μl) was incubated with CYP2B4 and phosphate buffer as outlined above. This protein solution was added to a colloidal solution, preacidified with 15μl ascorbic acid.

Raman spectra were obtained from aggregated colloid in a 1cm fluorimeter cell, using conventional 90° collection geometry. Spectra-Physics 2020/2045 Argon ion lasers provided 457.9nm and 514.5nm exciting radiation. Scattered radiation was analysed by an Anaspec-modified Cary 81 scanning monochromator. Data acquisition used a Peltier cooled photomultiplier tube [Thorn EMI 9658R] and photon counting electronics [Anaspec Series 2000 Quantum Photometer]. Spectra were obtained with 100mW irradiation. A scanning rate of 1 cm^{-1} s^{-1} and slit widths of 5cm^{-1} (457.9nm) and 3.6cm^{-1} (514.5nm) corresponding to a resolution of 2cm^{-1}, were used.

RESULTS

Substrate-free CYP2B4

SERR spectra of substrate-free cytochrome P450 CYP2B4 are presented in Figure 1 (457.9nm excitation) and Figure 2(514.5nm irradiation). The symmetrical nature and sharpness of ν_3 (1502cm^{-1}) in Figure 1 indicates that the haem moiety is predominantly in one form and exists as a six coordinate and low spin **[6cls]** species. The positioning of the sensitive oxidation marker band, ν_4, at 1372cm^{-1} confirms that the iron in the cytochrome is in the ferric state. A small sharp band appears at 1482cm^{-1}. Its frequency is consistent with a six coordinate high spin **[6chs]** contribution to the ν_3 mode. The spin state sensitive mode ν_2 positioned at 1579cm^{-1} further confirms the presence of a **6cls** species. The low energy shoulder of this band may suggest some residual high spin haem but the size of the band is obscured by the presence of ν_{11} from the low spin form. The broad peak centred at 1629cm^{-1}, with a shoulder at 1622cm^{-1}, originates from the stretching of the vinyl substituents on the haem. Other modes which can be assigned in the 457.9nm **SERR** spectrum include the formally Raman forbidden modes, ν_{37} and ν_{38} at 1601cm^{-1} and 1548cm^{-1} respectively, vinyl in-phase scissoring mode at 1433cm^{-1} and a ν_{29} mode at 1401cm^{-1}.

Further analysis of substrate-free CYP2B4 by excitation near the Q bands (514.5nm) supports the existance of a six coordinate low spin species adsorbed on the colloid. The band at 1638cm^{-1} has been attributed to the **6cls** configuration of ν_{10}. Other modes present include vinyl modes, (1629cm^{-1}, 1622cm^{-1} shoulder), ν_2 (1583cm^{-1} ;**6cls**), ν_{38} (1548cm^{-1}; **6cls**), ν_{29} (1402cm^{-1}) and ν_4 at 1372cm^{-1}.

Substrate-Bound CYP2B4

Upon aminopyrine addition (0.9mM final concentration) substantial low to high spin conversion was noted (Figure 3A). The broadening of ν_3, and the position of the peak at 1494cm^{-1},

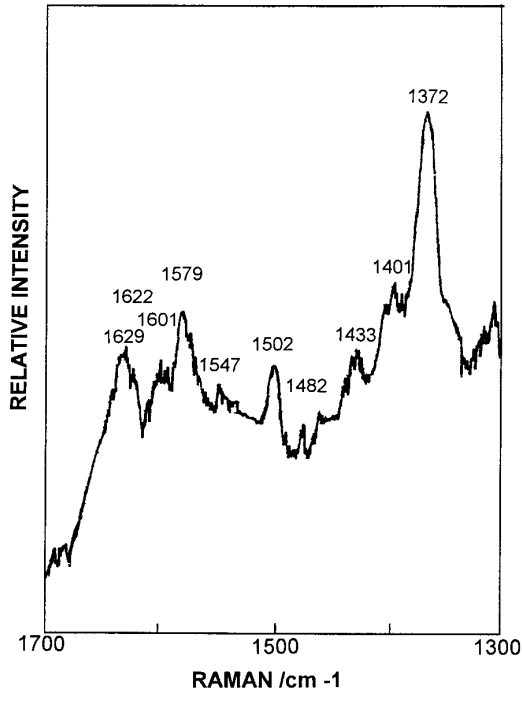

FIG. 1. SERR spectrum of P-450 CYP2B4 (1.30×10^{-7}M) recorded 30 minutes after colloidal aggregation.Exciting line: 457.9nm, 100mW.

reflects a major contribution of a **5chs** (five coordinate high spin) species. The high energy shoulder at 1625cm^{-1} is attributed to the high spin ν_{10} component. The broad scattering in the high energy region of ν_3 ($\sim$1502cm^{-1}) signifies retention of **6cls** protein on the surface, in the presence of excess substrate. Other modes present include ν_{37} (1599cm^{-1}), ν_{10} (1636cm^{-1})and a coplanar vinyl vibration at 1622cm^{-1}.

Following addition of laurate (Figure 3B), ν_3 is sharp and narrow suggesting a single haem form. In addition the frequency of 1494cm^{-1} reflects a greater degree of **6cls-5chs** conversion than that observed for aminopyrine as does the downfield shift of ν_2, observed at 1571cm^{-1}. However, a high energy shoulder at 1502cm^{-1} indicates that complete conversion of **6cls** has not occurred. The emergence of ν_{29} at 1400cm^{-1} arises from fatty acid binding within the pocket (see discussion). Other modes highlighted include the E_u mode ν_{37} (1597cm^{-1}) and vinyl stretches (1622cm^{-1}; 1629cm^{-1}).

DISCUSSION

Adsorption of the protein on the surface of silver colloidal particles is a necessary prerequisite for the **SERRS** phenomena. To ensure that the protein is adsorbed without denaturation or reaction with Ag ions, a colloid with a protective organic layer is used. The colloid produced is an almost monodispersed suspension of well defined hexagonal silver particles coated with a monolayer of chemisorbed citrate.This layer stabilises colloidal particles within the suspension and acts as a barrier, preventing direct protein to silver interactions [17]. Furthermore, it insulates the haem pocket from the metal surface and prevents haem extraction - a problem with borohydride-reduced silver colloids which have no protective organic layer [14].

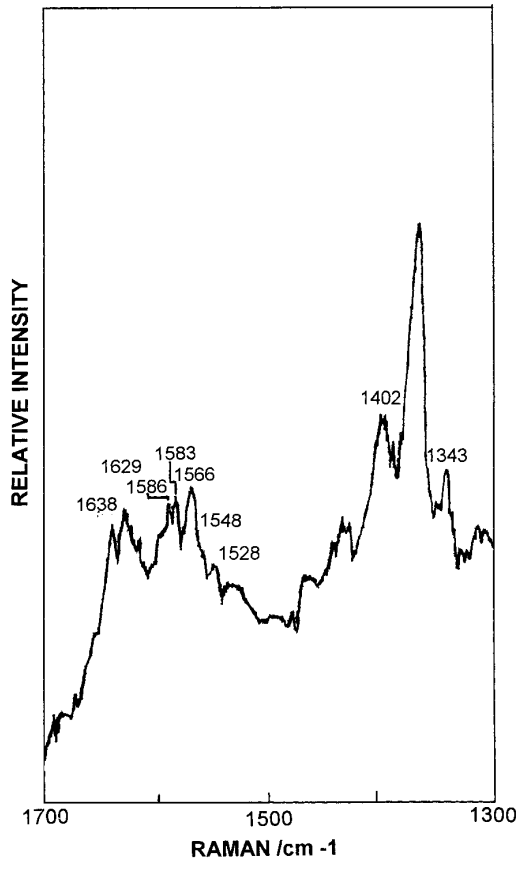

FIG. 2. SERR spectrum of P-450 CYP2B4 (6.53×10^{-7}M) recorded 30 minutes after colloidal aggregation. Exciting line: 514.5nm, 100mW.

Substrate-Free CYP2B4

The symmetrical nature and sharpness of ν_3 indicates that the haem moiety is predominantly in one form namely a **6cls** species.The presence of a single **6cls** species is in agreement with solution resonance Raman studies of cytochrome P450 CYP2B4 by *Hildebrandt* [25] which substantiated UV-Vis work by *Lange* [28] and his coworkers. No ν_3 band attributable to **5chs** could be discerned from the **SERR** spectrum, suggesting **6cls** to **5chs** conversion has been minimised upon protein adsorption onto the protected silver surface.

The observed **SERRS** activity of vinyl modes results from their coupling with the porphyrin π-system. The vinyl band is assymmetric due to the presence of two structurally different vinyl substituents on the haem. The lower frequency band arises from a vinyl group coplanar with the major haem plane whilst the higher energy band may arise from a vinyl group tilted with respect to the haem plane [24]. The dominance of the higher energy band in the **SERR** spectrum may be caused by the orientation of each vinyl group relative to the **SERRS**-active silver surface. A **6cls** mode for ν_{10} was not observed upon 457.9nm excitation either because it is weak due to surface selection rules for haem orientation or simply hidden under the dominant vinyl stretch positioned at 1629cm^{-1}.

As expected, symmetric vibrations are weaker in the preresonant **SERRS** obtained with

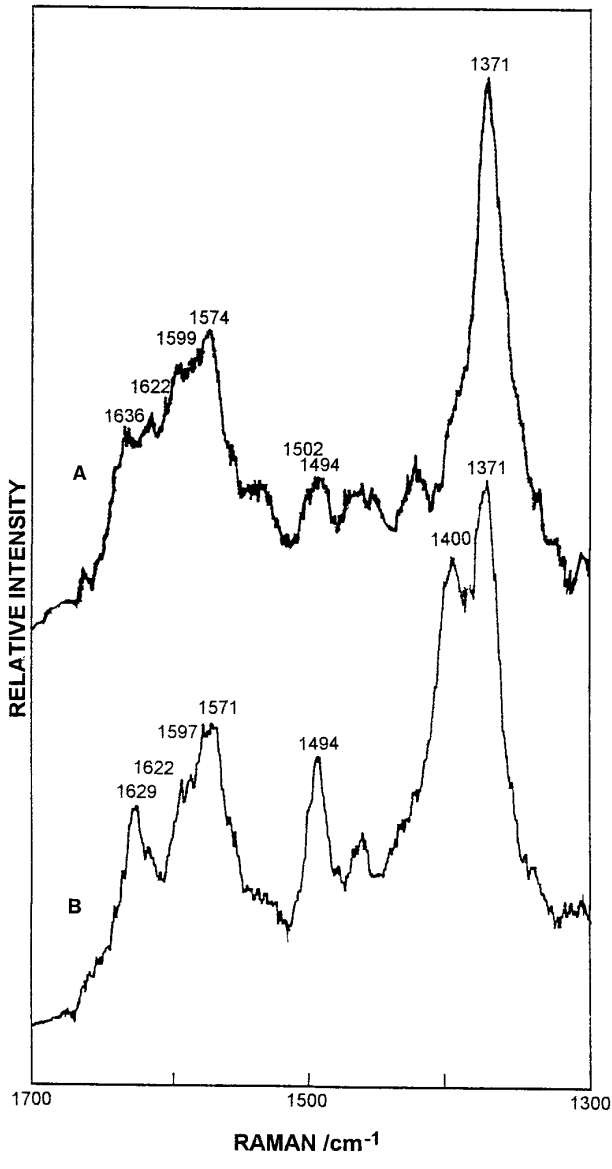

FIG. 3. Recorded SERR spectra of P-450 CYP2B4 in the presence of aminopyrine (A) (final substrate concentration 8.98×10^{-4}M: 1.19×10^{-7}M P-450) and in the presence of sodium laurate (B) (10.35×10^{-4}M: 9.44×10^{-8}M CYP2B4). Spectra obtained 30 minutes after colloidal aggregation. Exciting line: 457.9nm, 100mW.

514.5nm excitation. Both **6cls** and **6chs** ν_3 contributions in the 1480-1500cm^{-1} region are barely resolvable from background noise. Relative **SERR** intensity enhancement of other non-symmetric modes is shown by the emergence of ν_{11} (1566cm^{-1}; **6cls**) and modes attributed to A_{2g} bands ν_{20} (1343cm^{-1}; **6cls**) and ν_{19} (1586cm^{-1}; **6cls**). The broad band at about 1528cm^{-1} may be a ν_{38} **6chs** contribution. Only a minor contribution to the overall Raman signal occurs from vibrations due to **6chs** CYP2B4. This is indicated by the high frequencies observed for modes such as ν_{10}, ν_{19}, ν_2 and ν_{11}. These values reflect a contraction in the porphyrin ring

core [21]. Weaker axial binding, as in a **6chs** system, would enable haem expansion and the observation of lower frequencies for these modes.

All of these assignments indicate a **6cls** form of the haem and agree with studies of ferriprotoporphyrin IX model compounds [21] and the resonance Raman study of native cytochrome P450 CYP2B4 [23-25], ruling out major geometric alterations of the porphyrin on adsorption of the protein onto the 'biocompatible' silver surface.

Substrate-Bound CYP2B4

After aminopyrine addition, ν_3 shifts to 1494cm^{-1} from 1502cm^{-1}. This is characteristic of type-I substrate binding which alters the spin equilibrium towards the **5chs** form. The **6cls** ν_3 component appears as a shoulder at 1502cm^{-1}. Further support for **6cls** to **5chs** conversion is provided by a broad band positioned at 1574cm^{-1}. This peak (a superimposition of ν_2, ν_{19} and ν_{11}) is shifted downfield by 5cm^{-1} on substrate addition, demonstrating a greater contribution from high spin components.

Following addition of laurate (Figure 3B), ν_3 is sharp and narrow suggesting a single form of haem on the surface. This reflects a greater degree of **6cls** to **5chs** conversion than that observed for aminopyrine, as does the downward shift of ν_2, observed at 1571cm^{-1}. However, a high energy shoulder at 1502cm^{-1} (ν_3) indicates that complete conversion of **6cls** has not occurred.

Additional evidence for substrate presence within the haem pocket is obtained from the broadening of ν_2 region with concomitant narrowing of the vinyl stretch (1629cm^{-1}) band. This is particularly clear in the **SERR** spectrum with laurate present. This is explained by vibrational energy transfer as detailed by *Hildebrandt* [25] whereupon water expulsion from the active site leads to haem band narrowing/broadening. Application of crude curve fitting routines to the 1629cm^{-1} vinyl stretch yielded Full Width Half Height (FWHH) readings of 20cm^{-1} and 13cm^{-1} for substrate-free and laurate bound P450 CYP2B4. This 35% reduction in FWHH upon fatty acid binding cannot be attributed to error alone. At least part of the reduction is substrate induced. The expected broadening of the ν_2 and ν_{38} region is also clear in the **SERR** spectra of both substrates.

SERR intensity of ν_{29} is markedly different for the two substrate-bound proteins. This band is known to couple strongly with the in-plane vinyl scissoring motion and is sensitive to changes in the protein environment. Laurate is a substrate for both CYP2B4 and the bacterial cytochrome P450, BM3 (CYP102). Recent published NMR data [30] for BM3 haemoprotein domain only, indicated that laurate was positioned 7.6Å above the haem, an unusually long distance for a substrate. Parallel **SERR** studies on this bacterial cytochrome P450 [*Macdonald, I.D.G. et al,* unpublished data] have shown similar enhancement of ν_{29}. These two proteins may have similar hydrophobic regions for positioning of laurate in their respective binding pockets. Subtle spatial amino acid realignments upon fatty acid binding to this interior face appear to have been transmitted to peripheral protein/haem contacts resulting in ν_{29} enhancement. Thus, differences in the band intensity between the substrates and the native cytochrome P450 CYP2B4 may reflect subtle alterations in the haem pocket upon substrate binding which cause a perturbation of the haem [17]. Moreover, these differences appear to be substrate dependent, with the long chain fatty acid leading to ν_{29} activation. The relative enhancement of ν_{29} is more apparent with 514.5nm (not shown) where the intensity (laurate-bound) is larger than that of ν_4. No resonance Raman data on CYP2B4 has been published on fatty acid substrate interactions nor has comparative effects on substrate-induced haem strain been mentioned in the literature. This substrate-dependent observation is the first reported study of differing binding effects (to the pocket wall) of sterically dissimilar substrates inducing strain on the macrocyclic ring within the active site.

Further differences, which indicate alterations to the haem pocket and which are dependent on the substrate are noted for the E_u bands (ν_{37} and ν_{38}) and for the vinyl region between 1620-1630cm^{-1}. The small frequency shift for ν_{37} of 1-4cm^{-1} upon the addition of aminopyrine or laurate, coupled with intensity differences probably reflect subtle structural differences to the peripheral porphyrin environment upon addition of sterically different substrates. The shifts to lower energy on substrate addition are better illustrated by the ν_{38} mode where broadening is accompanied by significant shifts of 5cm^{-1} upon aminopyrine addition and 10-20cm^{-1} upon laurate addition to the protein. The large downshift for the latter case reflects a greater high spin contribution and/or subtle haem differences previously discussed.

There is a significant increase in overall scattering efficiency for the laurate study. This appears to indicate that the haem is positioned closer (relative to aminopyrine case) to the **SERRS**-active surface. A relative intensity increase for the vinyl mode at 1622cm^{-1} coupled with the emergence of a band at 1636cm^{-1} (ν_{10}; **6cls**) indicate peripheral structural differences associated with aminopyrine binding relative to laurate-bound protein. These spectral changes are not caused by direct binding by the substrate to the haem but by effects transmitted to the immediate protein environment of the macrocyclic ring by the substrate being present within the active pocket. These subtle structural differences will be important in the relative catalytic rates for these two substrates. The nature of substrate binding to the interior wall of the active site will influence the bioenergetics and kinetics of oxidation of these different substrates.

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