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Surface enhanced resonance Raman scattering as a probe of the spin state of structurally related cytochromes P-450 from rat liver

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Surface enhanced resonance Raman scattering (SERRS) was observed from structurally related drug-induced rat liver cytochromes P-450 adsorbed on a silver colloid. Careful control of pH and the sequence of addition of components to the sol is required to prevent protein denaturation at the surface due to conversion to P-450's biologically inactive form P-420 or haem loss. A low-spin P-450 (PB_{3a}), a mixed low- and high-spin P-450 (PB_{3b}) and a predominantly high-spin P-450 (MC_{1a}) were investigated. Spectra recorded in the 1300–1700 cm^{-1} frequency region, containing the oxidation state marker ν_4 at 1375 cm^{-1} (Fe^{3+}) and spin state markers ν_{10} (1625 cm^{-1}, high-spin; 1633 cm^{-1}, low-spin) and ν_{19} (1575 cm^{-1}, high-spin; 1585 cm^{-1}, low-spin) were used to differentiate between the spin states of the various forms of cytochrome P-450. As well as the established spin state marker bands, the intensity of a band at 1400 cm^{-1} appeared to depend on the high-spin content. Thus, with this method SERRS from silver colloids can be used to determine spin states of related cytochromes P-450 in dilute solution (10^{-8} M) and may be of value in studies of protein-substrate interactions.

Cytochrome P-450; SERRS; (Rat liver)

1. INTRODUCTION

Cytochromes P-450 are a multigene family of haem proteins central in the metabolism of drugs and foreign compounds [1]. There is an increasing amount of information on the primary amino acid sequences of these proteins and as a result, a growing need to understand how this relates to P-450 function and in particular to substrate specificity. At present there are very few diagnostic probes for the haem environment of mammalian cytochromes P-450 which enable changes in the nature of the iron protoporphyrin (IX) active site during reaction with substrates and during monooxygenation to be established. Here, we report the use of surface enhanced resonance Raman scattering (SERRS) from a silver sol to study these effects. This technique has the advantage that it can operate with dilute solutions (~10^{-8} M) and if correctly applied does not cause denaturation either by haem extraction or P-450 to P-420 conversion.

Mammalian liver P-450 is the terminal haemoprotein component of the cytochrome P-450-dependent monooxygenase electron-transport chain. This enzyme system is characterised by its broad specificity towards foreign compounds. To some degree this is explained by the multiplicity of the P-450 system. In the rat there may well be more than 30 forms of cytochrome P-450 [1].

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In this study, two phenobarbital-inducible forms of P-450 with 97% sequence homology (PB_{3a} and PB_{3b}) [PB_{3a} and PB_{3b} are equivalent to forms b and e described by Ryan et al. [3], respectively; MC_{1a} is equivalent to form d] but which exhibit very pronounced differences in substrate specificity (Wolf, C.R. et al., unpublished) were used along with one methylcholanthrene-induced form which was chosen because of its high-spin character (MC_{1a}) [2].

Resonance Raman spectroscopy is a powerful probe of the porphyrin environment at the active site and it has been used as a spin and oxidation state marker to study the relatively robust P-450cam [4] and liver microsomal cytochromes P-450 [5]. However, we have found that the technique requires large amounts of valuable enzyme. Other problems associated with this technique include fluorescence from the sample and denaturation of the protein. SERRS is inherently a more sensitive technique which has been used with cytochrome *c* at a silver electrode [6,7] and in a silver sol [8,9]. Here, we report the development of this technique for the study of the cytochrome P-450 haem environment, and potentially that of redox, spin state and other haem-related properties of this complex enzyme system, in a manner that can be applied practically.

2. EXPERIMENTAL

Sols were formed by boiling a solution of 90 mg silver nitrate in 500 ml distilled water with 10 ml of a 1% sodium citrate solution for 1 h. Sol pH varied between 6.5 and 7.0. 1% ascorbic acid was added to the sol, such that neutralisation to pH 7.0 could be achieved, after protein addition, by adding one drop of 0.1 M NaOH (~60–75 μl of 1% ascorbic acid to 2.9 ml sol). The pH of the sol on ascorbic acid addition was about 6. The amount of cytochrome P-450 added each time was 10 μl of ~20 μM. Neutralisation was carried out immediately. Cytochromes P-450 were purified using the procedures in [2,10]. The purity of the samples used were: 15.2, 15.1 and 15.1 nmol/mg protein for PB_{3a}, PB_{3b} and MC_{1a}, respectively. The samples were free from detectable amounts of the detergent Emulgen 911.

P-420, the biologically inactive form of P-450, was generated by the addition of sufficient 0.1 M NaOH to an aliquot of P-450 to achieve a final pH of 12.5–13.0. P-420 production was confirmed from the ferrous cytochrome absorption spectrum in the presence of carbon monoxide, the Soret absorption peak being shifted from 450 to 420 nm. P-420 was used in the same manner as described above for P-450. Raman spectra were recorded from sols held in a 1 cm cuvette flushed with cold nitrogen gas. 100 mW of 457.9 or 514.5 nm excitation, supplied by a Spectra Physics argon ion laser, was used and the Raman scattering was collected at 90° and detected using an Anaspec modified Cary 81 spectrophotometer with cooled photomultiplier tube and photon detection.

3. RESULTS AND DISCUSSION

Sols reported previously for SERRS [8,9,11] gave good signal-to-noise ratios with cytochrome *c* but caused rapid spin state conversion. However, we found that the sols reported in these experiments gave very poor results with rat liver cytochrome P-450 samples. Slightly acidic sols (pH 6) made with sodium citrate but not with sodium borohydride produced a large improvement with cytochromes P-450. This method has the advantage that, following protein addition, the sol may be neutralised, which appears to stabilise the protein. The 20% glycerol usually required to stabilise the cytochrome solution was not needed. Indeed, when a sol containing 20% glycerol was used, some spin state conversion was observed.

Of the three cytochromes P-450 investigated, one contained predominantly low-spin iron (PB_{3a}), one a mixture of high- and low-spin iron (PB_{3b}) and one predominantly high-spin iron (MC_{1a}) [2]. Raman and SERRS spectra of cytochromes in the high-frequency region (figs 1,2) contain the well known spin state markers ν_{10} (B_{1g}) and ν_{19} (A_{2g}) which are influenced by the $C_\alpha C_m$ stretching modes of the porphyrin ring [12,13]. ν_{10}, located between 1615 and 1640 cm^{-1}, is the most unambiguous spin state marker for haem proteins [14,15]. This peak is clearly resolved for all three proteins at 457.9 nm excitation (fig.1). For PB_{3a} the peak is at 1632 cm^{-1}, reflecting the low-spin nature of this protein. For PB_{3b} a shoulder at 1625 cm^{-1} as well as the band at 1632 cm^{-1} is observed indicating some high-spin haem. The high-spin nature of MC_{1a} is confirmed by the band at 1625 cm^{-1}. Con-

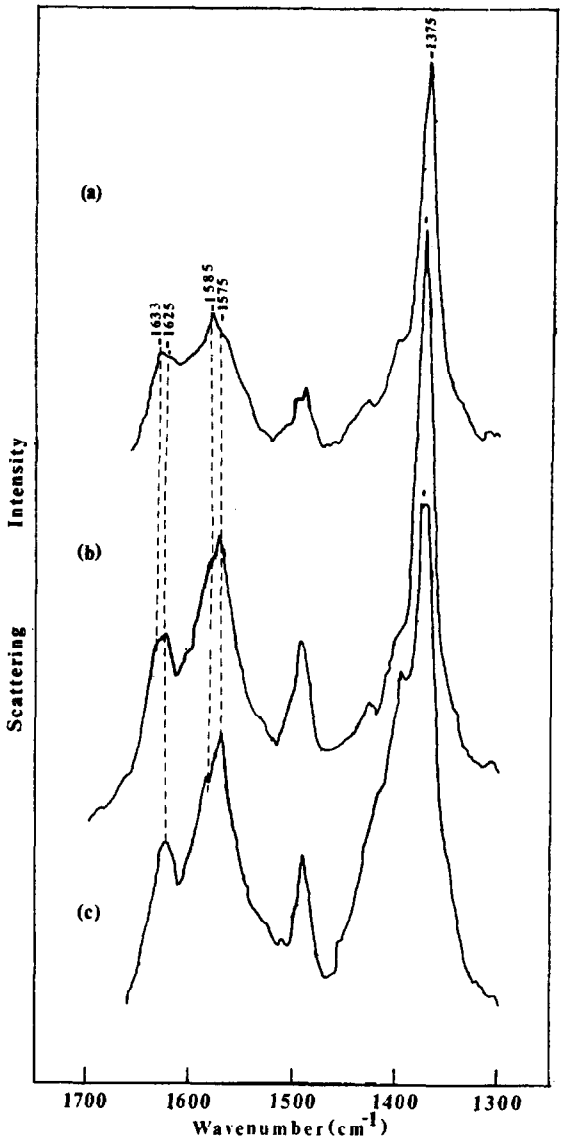

Fig.1. SERRS spectra of cytochrome P-450 with 457.9 nm laser excitation: (a) PB_{3a}; (b) PB_{3b}; (c) MC_{1a} on Ag sols. 100 mW laser power, 5 cm^{-1} slit width.

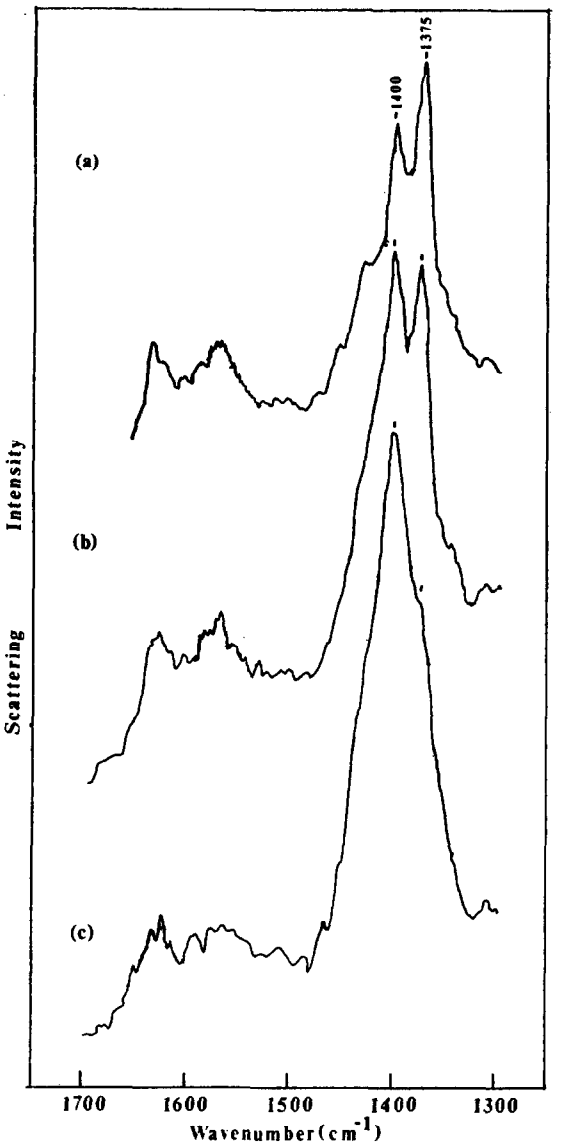

Fig.2. SERRS spectra of cytochrome P-450 with 514.5 nm laser excitation: (a) PB_{3a}; (b) PB_{3b}; (c) MC_{1a} on Ag sols. 100 mW laser power, 5 cm^{-1} slit width.

sistent behaviour is observed for ν_{19} (table 1), with corresponding relative intensity changes in 1585 cm^{-1} (low-spin) and 1575 cm^{-1} (high-spin) bands. Thus, the Raman spectra reflect the spin states, which are also observed in the UV-Vis spectrum. The oxidation state marker, ν_4 (A_{1g}), is assigned as the band at 1374 cm^{-1} and confirms that each protein is, as expected, in the ferric state.

The band at 1400 cm^{-1} was best resolved with 514.5 nm excitation. This band also clearly differentiates between the three proteins and has the advantage that its intensity can be readily compared with ν_4. The intensities follow the progression $MC_{1a} > PB_{3b} > PB_{3a}$ and may also reflect the spin states in these proteins. Close inspection of the MC_{1a} spectrum suggests the presence of two

Table 1

Vibrational frequency (cm^{-1}) peak positions from SERRS of cytochromes P-450 adsorbed on Ag sols

457.9 nm excitation			Mode	514.5 nm excitation		
PB_{3a}	PB_{3b}	MC_{1a}		PB_{3a}	PB_{3b}	MC_{1a}
1374	1375	1374	ν_4	1374	1376	1375
						1395
1400	1398	1398	ν_{20}/ν_{29}	1400	1402	1403
1432	1430	1430	δ_s (=CH_2)	1430		1425sh
1495	1494	1493	ν_3			
1502sh	1502sh	1502sh				
1574sh	1575	1573	ν_{19}	1575sh		
1582	1583sh	1586sh			1582	
						1594
1625sh	1625sh	1626	ν_{10}	1625sh	1625	1625
1633	1632			1634	1633	1633sh

sh, shoulder

bands at 1403 and 1397 cm^{-1}. Pairs of bands in this energy region have been assigned previously from resonance depolarisation data for other porphyrin systems [16] to ν_{29} (B_{2g}) at about 1405 cm^{-1} and ν_{20} (A_{2g}) at 1395 cm^{-1} and we assign the band at 1400 cm^{-1} as being due to this pair of modes being unresolved. It should be noted that Raman scattering from citrate and ascorbate on Ag sols can give signals in this energy region and it is possible that the band is a product of a complex interaction between these compounds and the protein. However, the intensity is clearly related to the specific enzyme sampled, irrespective of the origin of the effect. Whether this is a spin state marker alone or reflects other differences between the proteins as well remains to be resolved.

In order to establish the integrity of the protein adsorbed on the Ag sol surface, cytochrome P-450 was deliberately denatured to cytochrome P-420 (see section 2) the first likely stage in any denaturation process. Cytochrome P-420 spectra, observed with 457.9 nm excitation (not shown) are similar to those for cytochrome P-450. Appearance of a band at 1627 cm^{-1}, ν_{10} and the large relative intensity decrease of the 1400 cm^{-1} band system (fig.3) enables cytochrome P-420 to be clearly distinguished from cytochrome P-450 using 514.5 nm excitation. The position of ν_{10} suggests the presence of substantial high-spin haem indicating a spin state conversion associated with the denaturation.

The spectra for P-450 and its biologically inactive form P-420 are clearly different demonstrating that P-450 to P-420 conversion has been prevented with the sol conditions used. Further, from UV-Vis spectroscopy PB_{3a} and PB_{3b} are known to exist in low- and mixed low- and high-spin states respectively [2], the same states are observed using SERRS. Spin state would not be conserved if the proteins were denatured and the differences between these marker bands would be removed. This would be the case in either the formation of P-420 or with loss of haem, the other possible denaturation pathway of *b*-type cytochromes like P-450. Haem extraction has been observed with oxyhaemoglobin on sols produced by silver nitrate and sodium borohydride [8]. Band position and relative intensity changes accompany such denaturation as the free haem is adsorbed on the sol, possibly as the μ-oxo haem dimer. ν_4, the oxidation state marker, is shifted to a lower frequency of ~1370 cm^{-1} and the relative intensity of ν_3, at 1488 cm^{-1} for the μ-oxo dimer, increases appreciably [8]. Neither of these spectral properties was observed with the cytochromes P-450 adsorbed on the citrate sol, and thus haem loss does not occur at the sol surface.

Thus, SERRS allows the study of cytochrome P-450 proteins using small amounts of sample and can be used to determine specific oxidation and spin states of the iron and to differentiate between

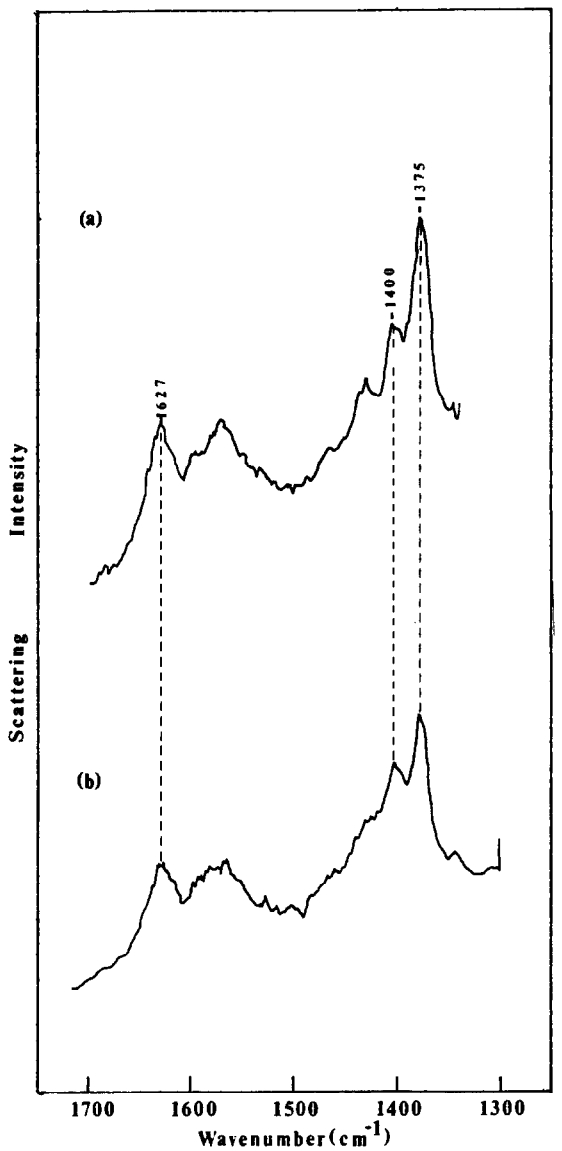

Fig.3. SERRS spectra of cytochrome P-420 with 514.5 nm laser excitation: (a) PB_{3a}; (b) PB_{3b} on Ag sols. 100 mW laser power, 5 cm^{-1} slit width.

P-450 and P-420. The 1400 cm^{-1} band not previously used in assessing the haem moiety is particularly useful for this purpose. The SERRS technique can be used to probe changes in the oxidation and spin state of cytochrome P-450 caused by manipulation of sol conditions. Using this method one can therefore approach the problem of establishing the importance of these parameters and the influence of the haem environment on cytochrome P-450 function. We are in the process of carrying out such studies.

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