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Surface enhanced resonance Raman study of phenobarbital-induced rabbit liver cytochrome P-450 LM2

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Surface enhanced resonance Raman (SERR) spectroscopy has been used to study the vibrational spectra of the heme of purified rabbit liver cytochrome P-450 LM2 which was adsorbed on colloidal silver suspensions or on a silver electrode. Based on a comparison with the resonance Raman (RR) spectra of the 'solute' species the high sensitivity of the SERR technique is demonstrated. Two different features were chosen in order to determine the structural and functional integrity of the adsorbed P-450. Both, substrate-induced spin state changes on the oxidized P-450 and the effect of the thiolate ligand on the oxidation state marker band ν_4 in the reduced P-450 could be observed in the SERR spectra of the adsorbed as well as in the RR spectra of the dissolved enzyme. These findings indicate that the protein structure near the substrate binding site and the coordination by thiolate are not affected by the interaction with the metal surface. Both structural elements are crucial for the function of P-450. Thus the elementary processes of the enzymatic action of P-450 can be investigated by this highly sensitive version of RR spectroscopy.

Enzyme structure; Benzphetamine; Enzyme-substrate binding; Surface enhanced resonance Raman spectroscopy; Cytochrome P-450; (Rat liver microsome)

1. INTRODUCTION

Mammalian cytochrome P-450 (P-450) catalyzes the oxygenation of a large variety of exogenous and endogenous compounds which involves oxygen activation by electron transfer to heme bound oxygen (review [1]). Among the various techniques which have been applied to elucidate the mechanism of the reaction cycle, resonance Raman spectroscopy can provide a very detailed picture of the heme structure and the heme-protein interactions, i.e. the active site of the enzyme [2–5]. In this work we have used surface enhanced resonance Raman spectroscopy which exhibits a much higher sensitivity than conventional RR spectroscopy [6]. SERR spectroscopy makes use of the enhancement of the RR scattering by up to six orders of magnitude when the molecules are adsorbed on roughened silver surfaces (electrodes, colloids). Thus, extremely low concentrations (10^{-7}–10^{-8} M) are sufficient to obtain high quality spectra. Furthermore this technique offers the advantage to quench the fluorescence of impurities which obscure the Raman signals in conventional RR spectroscopy. Finally, taking the metal/electrolyte interface as model system for charged biological membranes SERR spectroscopy can be used to monitor structural changes and reactions under the influence of the electrical double layer which may give new information about the mechanism of enzymatic reactions in vivo.

However, a necessary requirement for the application of SERR spectroscopy to P-450 is to find out if the native structure and function of the en-

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Abbreviations: bzph, benzphetamine; SERR, surface enhanced resonance Raman; RR, resonance Raman; $P\text{-}450_{Rec}$, cytochrome P-450 LM2 reconstituted in phospholipid vesicles; $P\text{-}450_{Em}$, cytochrome P-450 LM2 solubilized in Emulgen 913; $P\text{-}420_{Em}$, cytochrome P-420 LM2

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zyme are preserved after adsorption on the Ag surface. In the present paper we will discuss this problem based on SERR experiments of P-450 LM2 which has been purified from liver microsomes of phenobarbital-treated rabbits and reconstituted into phospholipid membrane vesicles ($P\text{-}450_{Rec}$) or solubilized in Emulgen 913 ($P\text{-}450_{Em}$).

2. MATERIALS AND METHODS

P-450 LM2 was isolated and purified as described elsewhere [7]. The enzyme was solubilized in 0.2% Emulgen 913 ($P\text{-}450_{Em}$) or reconstituted ($P\text{-}450_{Rec}$) in membrane vesicles of total microsomal lipid using a detergent (octylglucoside) dialysis technique [8]. Total microsomal lipid was extracted from rabbit liver microsomes [9] and further purified from other lipid membrane constituents (e.g. triglycerides, cholesterol) by column chromatography on silica gel. The samples contained 20%, v/v, glycerol and were buffered to pH 7.3 with potassium phosphate. The typical type I substrate bzph (synthesized according to [10]) was added to the protein up to saturating concentration. Reduction of the enzyme was achieved by adding a small amount of dithionite to the sample which was carefully degassed before. RR and SERR spectra were excited at 406 nm using conventional equipment which is described elsewhere [11]. The spectral band width was 5 cm^{-1}. SERR spectra were obtained from either colloidal silver suspensions (Ag sols) [12] or a rotating Ag electrode [13]. In both cases the bulk solutions contained 20%, v/v, glycerol, and, for $P\text{-}450_{Em}$, 0.2% Emulgen 913. The pH was adjusted to 7.3. In order to reduce photodecomposition or thermal heating by the exciting laser beam ($P_o \lesssim 30$ mW) the samples were continuously rotated in a quartz cuvette (0.3 ml sample volume) during the RR/SERR experiments. Before and after the RR experiments the integrity of the sample was checked by their absorption spectra according to [14].

3. RESULTS AND DISCUSSION

3.1. *RR spectra of $P\text{-}450_{Rec}$*

Fig.1A shows the RR spectrum of the oxidized $P\text{-}450_{Rec}$ (Fe^{3+}) in the absence of substrate. The poor signal-to-noise ratio is due to the strong background scattering of the vesicle suspension which was about 15-times higher than the peak height of the strongest RR band at 1372 cm^{-1}. The excitation line (406 nm) is in resonance with the maximum of the Soret absorption band of the heme chromophore so that totally-symmetric stretching vibrations of the tetrapyrrole-macrocycle are predominantly enhanced [15]. Thus the bands at 1372, 1500 and 1581 cm^{-1} can readily be assigned to the ν_4, ν_3 and ν_2 modes following the notation

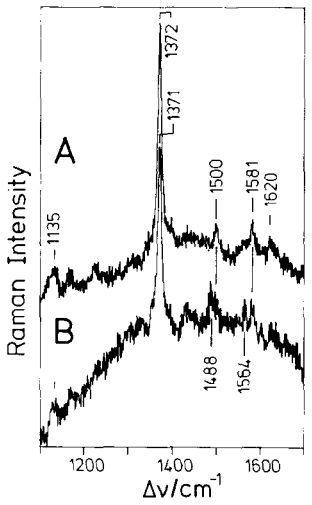

Fig.1. RR spectra of 10 μM $P\text{-}450_{Rec}(Fe^{3+})$ excited at 406 nm; 180 mM potassium phosphate buffer at pH 7.4, containing 20%, v/v, glycerol, 0.1 mM EDTA. (A) Without substrate; (B) complexed with benzphetamine (330 μM). Raman bands of glycerol and the background scattering are subtracted.

for porphyrin in-plane modes [16]. The peak at 1620 cm^{-1} results from the stretching vibration of the vinyl groups. Comparing RR and X-ray data of various porphyrin compounds linear relationships between the frequencies of the skeletal modes above 1450 cm^{-1} and the center-to-pyrrole-nitrogen distances (d_{core}) have been established [17]. Since d_{core} depends on the electron configuration of the metal (e.g. the effective radius) the frequencies are correlated with the spin and the ligation state.

The measured frequencies of ν_3 and ν_2 of the substrate-free $P\text{-}450_{Rec}(Fe^{3+})$ correspond very well with the six-coordinated low spin (LS) state which, according to EPR experiments [1], is the prevailing spin configuration. Binding of the bzph reveals a quite different spectrum (fig.1B). We note distinct new bands at 1564 and 1488 cm^{-1} which are assigned to ν_2 and ν_3 of the high spin (HS) configuration. The fact that these bands are comparable in amplitude with the LS state marker bands reflects a large amount of HS content which previously was estimated by absorption spectroscopy to 58% for the $P\text{-}450_{Rec}(Fe^{3+})$ –bzph complex [18]. The frequency of ν_3 which is in agreement with earlier results on microsomal P-450 solubilized in detergent [4] can be used to

distinguish between a six- and a five-coordinate HS state. This band is expected near 1480 and 1490 cm^{-1} for the six- and five-coordinate HS state, respectively [17]. Thus the measured frequency at 1488 cm^{-1} clearly points to a five-coordinate HS configuration. According to the linear relations in [17] this corresponds to a core-size radius of 2.02 Å [17]. The corresponding value derived from the ν_2 band at 1564 cm^{-1} is somewhat higher (2.03 Å) but still below the core-size for typical six-coordinated HS states (2.045 Å) [17].

3.2. *SERR spectra of P-450$_{Rec}$*

The SERR spectra of P-450$_{Rec}$(Fe^{3+}) in colloidal Ag suspensions reveal a much better signal-to-noise ratio (fig.2) than the RR spectra although the overall concentration is by a factor of 200 lower (50 nM). Since no RR bands can be detected from aqueous solution at such a low concentration the detected signals must originate from the adsorbed P-450$_{Rec}$(Fe^{3+}) whose RR scattering is enhanced via the coupling of the molecular oscillator and the vibrating free electrons of the metal [6]. The SERR spectrum of the substrate-free P-450$_{Rec}$(Fe^{3+}) in the spin state marker band region significantly differs from the corresponding RR spectrum. The mode ν_3 appears with two components, a strong band at 1491 and a shoulder at 1500 cm^{-1} indicating a dominant contribution of the five-coordinated HS configuration and a relatively small content of the LS form. The superposition of the marker bands ν_2, ν_{11}, and ν_{38} of both spin configurations may also account for the poorly resolved structure around 1578 cm^{-1}.

These spectral differences compared to the 'solute' P-450$_{Rec}$(Fe^{3+}) are not surprising. After adsorption at the Ag/electrolyte interface a large number of structurally quite different heme proteins undergo a partial transition from the six-coordinated LS to the five-coordinated HS state [12,19–21] which, in the case of cytochrome *c*, was shown to result in a thermal equilibrium between both spin configurations [12]. Evidence was provided that these structural changes are induced by direct electrostatic interactions between the electrical double layer and the iron porphyrin moiety and not mediated by the protein matrix [22]. This implies that the increase of HS content in the adsorbed P-450$_{Rec}$(Fe^{3+}) compared to the 'solute' state does not reflect a denaturation of the protein matrix. If, on the other hand, the native protein structure remains largely intact in the adsorbed P-450$_{Rec}$(Fe^{3+}) one would expect that substrate-binding produces similar structural changes as in the 'solute' state, i.e. a further shift of the spin state equilibrium towards the HS configuration. Indeed, the SERR spectrum of the P-450$_{Rec}$(Fe^{3+})–bzph complex (fig.2B) shows a relative decrease of the LS component of ν_3 at 1500 cm^{-1} with respect to the HS component at 1491 cm^{-1}. The frequency down-shift of the peak at 1578 cm^{-1} by 3 cm^{-1} can also be explained by an increasing contribution of the HS marker bands, specially of ν_2 at 1564 cm^{-1}.

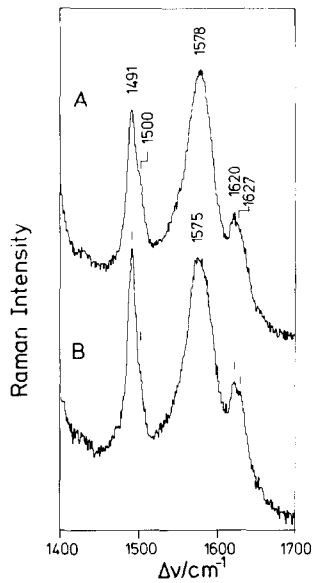

Fig.2. SERR spectra of 50 nM P-450$_{Rec}$(Fe^{3+}) on colloidal Ag at 406 nm (buffer and glycerol concentrations as in fig.1). (A) Without substrate; (B) complexed with benzphetamine. Raman bands of glycerol and the background scattering are subtracted.

The structural changes upon substrate-binding are also reflected by the SERR spectra in the low-frequency region (fig.3), which in the case of P-450$_{Rec}$ is not accessible for conventional RR spectroscopy due to the high background scattering. The main spectral changes are the intensity increase of the 254 cm^{-1} band and a small decrease of the band at 676 cm^{-1} compared to the doublet at 349 and 384 cm^{-1}. Furthermore, the frequency of the 720 cm^{-1} band shifts up by 4 cm^{-1}. In both spectra of the substrate-free and substrate-bound

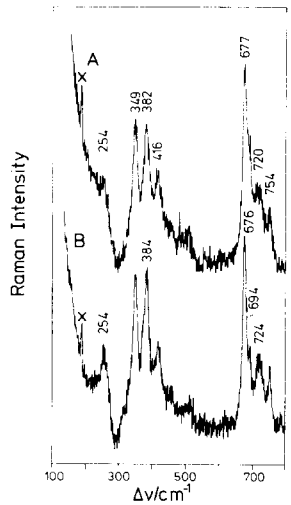

Fig.3. Low-frequency SERR spectra of P-450$_{Rec}$(Fe^{3+}) on Ag sol (same conditions as in fig.2). (A) Without substrate; (B) complexed with benzphetamine. Raman bands of the buffer and glycerol as well as the background scattering are subtracted. (×) Plasma lines of the exciting laser.

forms, there is no band at 398 cm^{-1}, which is regarded as a characteristic marker for the inactive form P-420 [23].

Thus, the SERR spectra of P-450$_{Rec}$(Fe^{3+}) and P-450$_{Rec}$(Fe^{3+})–bzph in the low- and high-frequency region demonstrate that the protein structure, as far as it influences the active and the substrate-binding site, is preserved after adsorption. Furthermore, the comparison with the RR spectra reveals the main advantage of the SERR technique: despite the extremely low concentration much better spectra can be obtained in a wide frequency range, even down to 100 cm^{-1}.

3.3. *RR and SERR spectra of the reduced P-450$_{Em}$*

A characteristic feature of the reduced P-450(Fe^{2+}) is the extraordinarily low frequency of the oxidation state marker band ν_4 at 1343 cm^{-1} [3]. This results from the strong π-back donation from the 5th ligand (thiolate) to the π^* orbitals of the porphyrin. This weakens the C-N-bonds whose stretching vibrations give the major contribution to ν_4 [15]. Thiolate as an axial ligand of the heme iron increases the electron density in the O-O bond of oxygen which is bound as the 6th ligand during the reaction cycle [24]. This effect is essential for the activation of oxygen. Thus the frequency of ν_4 in the O_2-free P-450(Fe^{2+}) which reflects the electron pushing effect of the thiolate ligand can be used as another sensitive marker for the functional integrity of the enzyme.

Fig.4B shows the RR spectrum of the reduced P-450$_{Em}$(Fe^{2+}) which was accumulated within 10 min by repetitive scanning. Three bands at 1343, 1359, and 1370 cm^{-1} can be detected in the region of the oxidation state marker band. The shoulder at 1343 cm^{-1}, which can be readily assigned to ν_4 of P-450$_{Em}$(Fe^{2+}), continuously decreased during the RR experiment until, after ~15 min, it could not be detected anymore. The disappearance of this band is accompanied by an increase of the 1359 and 1370 cm^{-1} bands.

Based on the absorption spectra (of the CO-complex) before and after the RR experiments it was found that the decay of the native P-450$_{Em}$(Fe^{2+}) corresponded to the conversion to the inactive form P-420 under the influence of the exciting laser beam. Thus, the RR bands at 1359 and 1370 cm^{-1} must be assigned to ν_4 of the reduced respective oxidized P-420. It should be mentioned that Ozaki et al. [3] reported similar observations in the RR spectra of P-450$_{Em}$(Fe^{2+}) excited at 488 nm although the photodegradation proceeded on a larger time scale. Apparently the

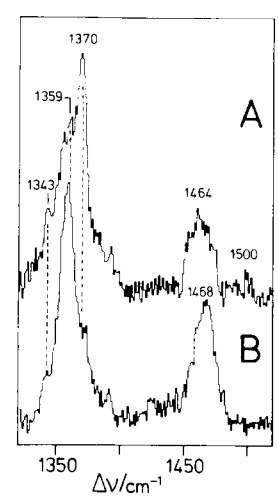

Fig.4. SERR and RR spectra of P-450$_{Em}$(Fe^{2+}) excited at 406 nm (buffer and glycerol concentrations as in fig.1). (A) SERR, Ag electrode, −0.65 V; 0.1 M Na_2SO_4; 100 nM P-450$_{Rec}$; (B) RR, aqueous solution, 10 μM P-450$_{Rec}$(Fe^{3+}). The 1464 and 1468 cm^{-1} bands result from the adsorbed and dissolved glycerol, respectively.

photolability is enhanced when the excitation wavelength is tuned to the maximum of the Soret absorption. In order to obtain a SERR spectrum of P-450$_{Em}$(Fe^{2+}), the enzyme was adsorbed on the Ag electrode at a potential of −0.65 V which is sufficient for a complete reduction [25]. The SERR spectrum (fig.4A) which was recorded under the same conditions as under in the RR experiment is similar to the RR spectrum. Again we note three oxidation state marker bands (ν_4) at the same positions (1343 cm^{-1}: P-450$_{Em}$(Fe^{2+}); 1359 cm^{-1}: P-420$_{Em}$(Fe^{2+}); 1370 cm^{-1}: P-420$_{Em}$(Fe^{3+})) and the 1343 cm^{-1} band gradually disappearing on the same time scale as in the RR experiment. This implies that the same molecular processes underlying the photoinduced conversion of P-450 to P-420 occur in the adsorbed and in the dissolved state. However, the intensity distribution between the 1359 and 1370 cm^{-1} SERR bands implies that a larger part of P-420$_{Em}$(Fe^{2+}) is reoxidized on the Ag electrode than in the solution. Apparently, the redox potential of P-420$_{Em}$ is more negative than −0.65 V.

The comparison of the RR and SERR spectra provides evidence that the thiolate-iron bond in P-450 remains intact after adsorption on the Ag electrode. This implies that an essential structural feature for oxygen activation is preserved in the adsorbed enzyme. The photolability of P-450$_{Em}$(Fe^{2+}) which is found in both the adsorbed and the 'solute' form is an intrinsic property of this specific state and is not affected by the interaction with the metal surface.

4. CONCLUSIONS

It was demonstrated that the structure of microsomal P-450, in particular, the substrate-binding site and the thiolate-iron linkage remain intact after adsorption at the Ag/electrolyte interface. Both structural elements are essential for the function of P-450 so that it is reasonable to assume that the catalytic activity of the adsorbed enzyme is preserved. This would be in line with the successful application of P-450 in electro-enzyme reactors (review [26]). Thus further systematic investigations by SERR spectroscopy are justified to elucidate structure-function relationships during the reaction cycle of P-450.

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