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Active sites of two orthologous cytochromes P450 2E1: Differences revealed by spectroscopic methods

Eva Anzenbacherova [a], Jiri Hudecek [b], Daniel Murgida [c], Peter Hildebrandt [c], Stéphane Marchal [d], Reinhard Lange [d], Pavel Anzenbacher [e,*]

[a] Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacky University, CZ-775 15 Olomouc, Czech Republic
[b] Department of Biochemistry, Faculty of Sciences, Charles University, CZ-128 40, Prague 2, Czech Republic
[c] Max-Volmer-Laboratory for Biophysical Chemistry, Technical University Berlin, 10623 Berlin, Germany
[d] INSERM U710, University of Montpellier 2, 34095 Montpellier Cedex 5, France
[e] Institute of Pharmacology, Faculty of Medicine, Palacky University, CZ-775 15 Olomouc, Czech Republic

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Abstract

Cytochromes P450 2E1 of human and minipig origin were examined by absorption spectroscopy under high hydrostatic pressure and by resonance Raman spectroscopy. Human enzyme tends to denature to the P420 form more easily than the minipig form; moreover, the apparent compressibility of the heme active site (as judged from a redshift of the absorption maximum with pressure) is greater than that of the minipig counterpart. Relative compactness of the minipig enzyme is also seen in the Raman spectra, where the presence of planar heme conformation was inferred from band positions characteristic of the low-spin heme with high degree of symmetry. In this respect, the CYP2E1 seems to be another example of P450 conformational heterogeneity as shown, e.g., by Davydov et al. for CYP3A4 [Biochem. Biophys. Res. Commun. 312 (2003) 121–130]. The results indicate that the flexibility of the CYP active site is likely one of its basic structural characteristics.

Keywords: Cytochrome P450; CYP2E1; Active site; Resonance Raman; Absorption spectroscopy; High pressure

Cytochrome P450 2E1 (CYP2E1) is an enzyme known to be expressed in liver as well as in extrahepatic tissues as, e.g., brain, lungs or lymphocytes. Its endogenous function remains to be elucidated, although there are clues indicating its involvement in lipid and glucose metabolism [1]. CYP2E1 belongs to one of the first liver microsomal cytochromes P450 isolated and its role in metabolism of relatively small xenobiotics (as ethanol, anesthetics, and solvents) as well as in activation of carcinogens (nitrosamines) has been widely studied. In the absence of crystallographic data for this enzyme, discussions of structure–function relationships rely on models based on known structures of other CYP forms [2,3].

Active sites of liver microsomal CYP enzymes should be able to accommodate a variety of substrates, however maintaining a certain degree of specificity. Recent crystallographic analyses have shown that the volume of the active site, i.e., the space available for a substrate is not the only factor determining the ability of a substrate to be present in the active site. Surprisingly, the CYP3A4 enzyme crystal structure has been shown to possess a small active site, although the substrates of this enzyme are molecules with MW up to 1 kDa [4,5]. The key to understanding this seeming discrepancy may be in the flexibility of the enzyme active site. In fact, the apparent compressibility (determined from the redshift of the Soret absorption band with hydrostatic pressure [6]) of the CYP3A4 heme pocket in the presence of substrate has been shown to be relatively high among CYP enzymes

* Corresponding author. Fax: +420 58 563 2569.
E-mail address: anzen@tunw.upol.cz (P. Anzenbacher).

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[7]. Also, resonance Raman data could be interpreted in terms of a more flexible heme pocket in this enzyme [8].

Here, the focus is given to comparison of the active site properties of two CYP2E1 proteins, namely, of the human recombinant enzyme and of its minipig liver microsomal counterpart. Although these proteins exhibit similar enzyme activities as well as a high degree of identity of the primary structure (minipig enzyme is 79.6% identical to the human form [9]), their active sites apparently differ in flexibility.

Materials and methods

Materials. All chemicals (if not stated otherwise) were obtained from Sigma–Aldrich (Sigma–Aldrich CZ, Prague). CYP2E1 enzymes were either purchased from Panvera (human recombinant, Panvera-Invitrogen, Carlsbad, CA) or isolated from minipig liver by chromatographic methods as described previously [9]. Enzyme activities of both CYP2E1 (chlorzoxazone 6-hydroxylation and *p*-nitrophenol hydroxylation) in a reconstituted system [10] were checked to confirm that both enzymes exhibit activities typical of the native enzyme.

Methods. Absorption spectra of the dithionite-reduced CYP2E1 under hydrostatic pressure were taken with a Cary 3E (Varian, Palo Alto, CA) spectrophotometer equipped with a high-pressure cell with sapphire windows placed in the sample compartment. Details of the instrument as well as of the applications of this method to studies of P450 enzymes have been published elsewhere [11,12]. CYP2E1 enzymes were diluted to a concentration of about 2 μM by 20 mM K/phosphate buffer, pH 7.4, containing 20% glycerol. Chlorzoxazone concentration was 50 μM.

RR spectra were obtained by the 413-nm line of a Kr^+ ion laser (Coherent 302) with a laser power below 8 mW at the sample. For the RR experiments, the proteins were dissolved in the phosphate buffer described above with a concentration of ca. 10 and 15 μM for the minipig or human enzyme, respectively. The chlorzoxazone concentration was 100 μM. The samples were measured at −140 °C using a Linkam cryostat (THMS 600, Waterfield, UK) that was adapted to a confocal Raman spectrometer (LabRam HR800, Dilor) equipped with a liquid nitrogen-cooled CCD camera. All spectra data were processed using Sigma Plot software (version 8.0, SPSS, Chicago, IL) and Origin (ver. 6.0, OriginLab, Northampton, MA).

Results

Absorption spectra under high pressure

Difference spectra in Figs. 1A and B (the reduced enzyme in complex with carbon monoxide *minus* the spectrum of reduced form) show the dominant spectral absorption band at 450 nm characteristic for cytochromes P450 and a minor band/shoulder at about 420 nm (its exact position is, in part, obscured by the background slope), attributable to a small fraction of the inactive P420 form or to differences in reduction in the presence and absence of CO. As the hydrostatic pressure increases (curves 1–4), a redshift of the P450 absorbance maximum is observed together with the increase in the fraction of the inactive cytochrome P450 form, P420 (corresponding to the spectral maximum at about 420 nm). The human CYP2E1 (Fig. 1A) is apparently more susceptible to the P450–P420 transition; after increase of pressure to 200 MPa, it is mostly (and irreversibly) converted to the inactive P420 form. The absence or presence of substrate (chlorzoxazone) does not induce any significant changes in this respect (data not shown). On the other hand, the minipig enzyme is rather stable even at 300 MPa with the inactive P420 form reaching only about 30% of the total enzyme content (Fig. 1B). The conversion is largely irreversible, as the fraction of P420 remains stable after the hydrostatic pressure is released back to 0.1 MPa (curve 5).

Redshift of the characteristic P450 Soret band upon application of high hydrostatic pressure has been shown to reflect the compressibility of the heme pocket.

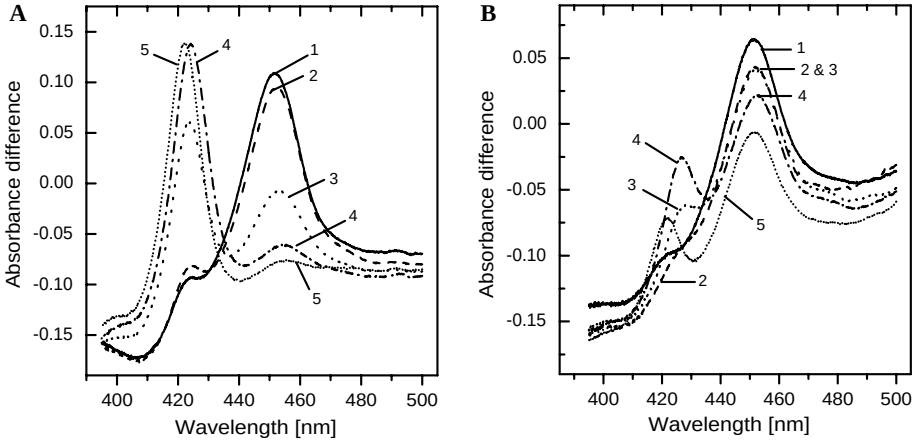

Fig. 1. Absorption spectra of human (A) and minipig (B) CYP2E1 enzymes at increasing hydrostatic pressure. Difference spectra of the reduced CO complexes of CYP2E1 (vs. the reduced sample) taken in the presence of substrate chlorzoxazone; pressure: 0.1 MPa, traces 1, full line, 100 MPa, traces 2 (dashed), 200 MPa, traces 3 (dotted), 300 MPa, traces 4 (dashed and dotted line), pressure release to 0.1 MPa, and traces 5 (finely dotted line).

Position of this band is directly related to changes in microscopic dielectric constant of the heme surroundings due to altered arrangement or expulsion of water molecules caused by pressure increase [6,11]. The slope (α) of the linear dependence of the Soret band position (expressed as wave number) on the applied pressure is related to the compressibility of the chromophore environment, which is the heme moiety of the hemoprotein [6]. This analysis was applied first to P450cam (CYP101) [6]; further studies have shown that the compressibility of cytochrome P450 active sites varies significantly among different forms [7]. The data obtained in the same way for human and minipig CYP2E1 both in the presence and in the absence of substrate (chlorzoxazone) are presented in Table 1 together with values found for other CYP enzymes for comparison. Results obtained here show that the two CYPs studied, although being formally classified identically as CYP2E1, differ in compressibility of their heme active sites. Interestingly, the larger susceptibility of the human CYP2E1 to the P450–P420 transition may be caused by the greater compressibility of its heme active site reflected by the larger values of the coefficient ($-\alpha$) for the human enzyme (see Table 1). Minipig CYP2E1 resembles, in this respect, the human CYP1A2 or the bacterial CYP101 (P450cam) enzymes.

Resonance Raman spectra

Resonance Raman spectra of both human and minipig CYP2E1 display the features characteristic of P450 enzymes [13]. In the high-frequency region (Fig. 2), the strong ν_4 band at about 1373 cm^{-1} dominates. Despite the overall similarity, the spectral traces for both forms indicate some subtle structural differences. The spectral interval 1450–1650 cm^{-1} is known to contain several bands sensitive to the heme iron spin state and porphyrin structure (“core-size marker bands”). An enlargement of this part of the spectra is shown in Fig. 3. The

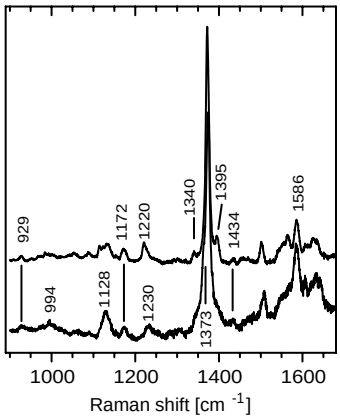

Fig. 2. High-frequency region of the resonance Raman spectra of CYP 2E1 enzymes. Human CYP2E1 (upper trace), and minipig CYP2E1 (lower trace), both in substrate-free oxidized resting state, excitation line 413.1 nm, frozen solution (−140 °C), and laser power below 6 mW.

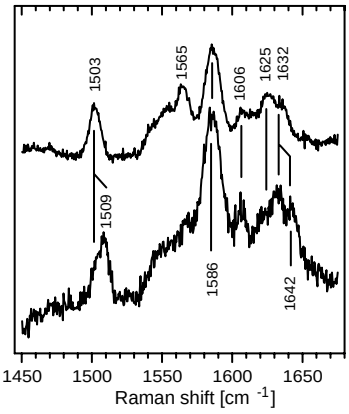

Fig. 3. Enlargement of the marker band region of the high-frequency part of the Raman spectra, as in Fig. 2.

spin state marker band ν_3 is located at 1503 cm^{-1} in the human enzyme, in a position typical of CYPs with low-spin heme iron. Also the other marker bands are

Table 1
Soret band shift data for reduced complexes with carbon monoxide of the respective CYP2E1 enzymes in the presence and absence of substrate chlorzoxazone; data for CYP3A4, CYP1A2, and CYP101 included for comparison

Enzyme	$-\alpha$ (cm^{-1}/MPa)	S_0 (cm^{-1})	Correlation coefficient
CYP2E1 human	0.391 ± 0.003	22,149	0.9885
CYP2E1 human + substrate	0.440 ± 0.010	22,144	0.9634
CYP2E1 minipig	0.220 ± 0.004	22,119	0.9885
CYP2E1 minipig + substrate	0.172 ± 0.009	22,151	0.9754
CYP3A4[a]	0.246 ± 0.009	22,365	0.9881
CYP3A4 + substrate TAO[a]	0.341 ± 0.001	22,396	0.9978
CYP1A2[a]	0.253 ± 0.003	22,371	0.9937
CYP101[b]	0.272 ± 0.007	22,371	0.9943
CYP101 + substrate *R*-camphor[b]	0.160 ± 0.007	22,433	0.987

Analysis of the absorption spectra was carried out according to [6]; Soret band shift follows the equation $S = S_0 + \alpha \cdot P$, where S is the Soret band position at given pressure P, S_0 is its position at zero pressure, and slope α is related to compressibility.

[a] Ref. [7].

[b] Ref. [6].

located at positions very similar to those reported for other P450 enzymes in the low-spin state (ν_2 at about 1586 cm^{-1}, ν_{10} as a broad feature 1632–1636 cm^{-1}) in the human enzyme.

However, in the minipig form the ν_3 band is apparently shifted to a higher position, and a closer inspection reveals the presence of two bands. This was confirmed by peak fitting procedure, resulting in two bands located at 1503 and 1509 cm^{-1} (Table 2). The stronger band at 1509 cm^{-1} is in a position previously not observed for cytochromes P450. In addition, also the ν_{10} band is visibly split to two components at 1632 and 1642 cm^{-1}. Such an upshift of the marker bands (ν_3, ν_{10}) may be attributed to a reduction of the heme nonplanarity, as their shifted positions (1509, 1642 cm^{-1}, respectively) are similar to those observed for planar hexacoordinated low-spin model compounds [14]. The Raman evidence for a presence of two different heme conformations in the minipig enzyme is supported by the high-pressure experiment (Fig. 1B), where the enzyme divides in a similar proportion to the P450 and P420 forms. For CYP3A4, a conformational heterogeneity was reported recently [15] by Davydov et al., who found a presence of two conformational subsets, differing in reduction potential. As the deviations from heme planarity are known to strongly influence this property [16], the differences in deformation degree of the heme found in this work may offer one plausible explanation of such a heterogeneity. The fact that the Raman spectra were measured at a lowered temperature in this work possibly aided to preserve the more restricted (“planar”) conformer against photochemical conversion.

Comparison of the spectra in the high-frequency region reveals additional differences in relative intensities of several vibrational bands. The ν_{11} band seen at 1565 cm^{-1} in the human enzyme (Fig. 3) is present only as a shoulder in the spectra of the minipig form, possibly also due to frequency upshift and splitting. Similarly, the well-resolved bands in the spectrum of human enzyme at about 1340 and 1395 cm^{-1} are present only as shoulders in the spectrum of the minipig variant. These bands may be assigned to ν_{41}, ν_{12} modes [17] or, alternatively, to ν_{20}, ν_{29} vibrational modes [13]. As these modes gain intensity with Soret excitation only due to the lowering of heme symmetry, the observed intensity differences are in line with the increase in heme planarity of the minipig enzyme.

In the low-frequency part of the spectra (Fig. 4), the modes between 340 and 450 cm^{-1} are known to be strongly influenced by mode mixing. Nevertheless, the band located at about 346 cm^{-1} was assigned to the porphyrin skeletal ν_8 mode, the band at about 377 cm^{-1} to the bending mode of heme propionates, and for the features above 390 cm^{-1}, the contribution of vinyl side chains is dominant [17,18]. As we have described previously [19], the cytochromes P450 display two distinct spectral patterns in this region: the first group (CYP3A4, 1A2, 2B4, 51, 101) has no resolved band between the propionate mode and the 420 cm^{-1} vinyl band, whereas the enzymes of the second group (CYP102, 11A1, and oxygenase domain of nNOS) have additional band(s) at about 390 cm^{-1}. Interestingly, while the human CYP2E1 belongs to the first category, the minipig enzyme obviously not. This difference may reflect either a conformational heterogeneity of vinyl substituents in 2- and 4-positions, or some restriction in vinyl mobility, as suggested by the comparison with spectra of myoglobin [17] and cytochrome *c* [18], where the corresponding groups are covalently linked to the apoprotein via thioether bonds. On the other hand, an influence of deviations from planarity on these modes is also probable.

There are noticeable spectral differences between both enzymes in the wave number interval 450–900 cm^{-1}, otherwise dominated by the in-plane porphyrin skeletal ν_7 mode at about 674 cm^{-1}. Most of the low-frequency bands, differing in their positions and relative intensities in both forms, may be assigned to the out-of-plane porphyrin modes [17,18]: γ_{22} (~490 cm^{-1}), γ_{12} (~505 cm^{-1}), γ_{11} (~718 cm^{-1}), γ_5 (~728 cm^{-1}), γ_1 or γ_{15} (~751 cm^{-1}), γ_4, and γ_{10} or γ_{19} (≡820 and 840 cm^{-1}, respectively). The

Table 2
Results of the peak fitting procedure for the Raman spectra of minipig CYP2E1 (Lorentzian peak shape, linear background, and wave number interval 1465–1530 cm^{-1})

Peak position (cm^{-1})	Halfwidth (cm^{-1})	Area (arbitrary units)
1503.2 (0.6)	7.8 (1.5)	0.69 (0.18)
1509.1 (0.3)	6.2 (0.8)	0.91 (0.16)

Values in parentheses are error estimates.

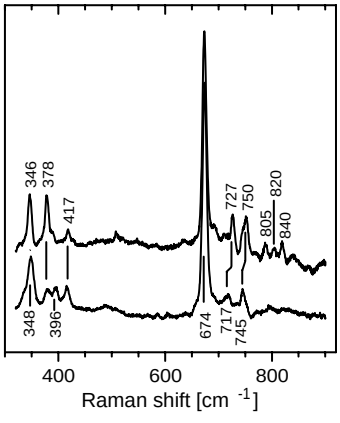

Fig. 4. Low-frequency region of the resonance Raman spectra of CYP2E1 enzymes. Human CYP2E1 (upper trace), and minipig CYP2E1 (lower trace), both in substrate-free oxidized resting state, excitation line 413.1 nm, frozen solution (−140 °C), and laser power below 8 mW.

remaining bands in the low-frequency spectra of human enzyme (at about 788 and 804 cm^{-1}) have been found in resonance Raman spectra of several CYPs and assigned to skeletal ν_{32} and ν_6 modes [13]. The out-of-plane vibrational modes are not expected to be enhanced in resonance with the B and Q bands of the heme, and their appearance in the spectra can be interpreted as a sign of a release of the symmetry selection rules due to the interaction of heme with the apoprotein. Therefore, presence of several bands of this category in the spectrum of the human form may be well taken as an additional indication of the larger flexibility of this form in comparison with the minipig enzyme, where the conformationally restricted (more planar) heme conformer is stabilized by the protein.

Discussion

This work deals with active site properties of two enzymes which are classified both as CYP2E1 according to the systematic nomenclature of cytochromes P450. Their primary structures are 79.6% identical (human CYP2E1, PubMed Accession No. P05181, minipig CYP2E1 GenBank Accession No. AY581116). Comparison of the regions purported to be involved in substrate recognition [2,3,9] did not reveal any prominent differences in the amino acid structure. On the other hand, human and minipig CYP2E1 differ in the primary structure in positions with a potential to influence the properties of the regions close to the heme, and, moreover, which may be important for positioning of the helices forming the architecture of the protein (helices D, K, and K′). For example, the His208 involved in the helix F and Asn135 of the helix C close to the heme are changed in the minipig enzyme to Tyr and to Asp, respectively, the Gln345 at the beginning of the helix K has a counterpart in a Leu residue, while another Gln161 at the end of D helix is replaced by His in the minipig enzyme.

The extent of conversion of the CYP enzymes to their inactive form, P420, in other words, the relative susceptibility with which the CYP enzymes are inactivated under hydrostatic pressure, varies from one CYP enzyme to another; moreover, factors from the surrounding medium (as the presence of phospholipids or of glycerol in the buffer) have been shown to play an important role [7,15]. In the present work, all data were collected in buffer containing glycerol and at the same conditions. Among CYP enzymes inactivated relatively easily by the hydrostatic pressure are CYP3A4 [7,15], CYP2B4 [20] and, as shown here, also the human CYP2E1. On the other hand, relatively stable are (under conditions described) CYP1A2 [7] and the minipig CYP2E1, as well as the microbial soluble enzymes CYP101 [6] and CYP102 [7].

The flexibility of the active site seems to be one of its basic characteristics. In fact, this ability is possibly of general importance, as it may help proteins to accommodate various substrates and may be a complementary feature to sterical and 'chemical' demands of their active sites. The different behavior with respect to the P450 to P420 transition is likely to be caused by differences in protein–heme contacts and interactions. (Because of the primary structure homology, a simple dissimilarity in apoprotein stability is less probable, at least in our case of two CYP2E1 forms.) Also, the differences in resonance Raman spectra of both CYP2E1 samples point to such influences of the apoprotein on the heme moiety. This may represent one of the general mechanisms of modulation of P450 enzyme properties.

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