# HIGH CONFORMATIONAL STABILITY OF CYTOCHROME P-450 1A2. EVIDENCE FROM UV ABSORPTION SPECTRA

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The fourth derivative of absorption spectra between 260 and 310 nm were used for monitoring the changes in exposure of tyrosine and tryptophan side chains in cytochrome P-450 1A2 to solvent. Titration of the enzyme with a specific inhibitor, α-naphthoflavone (2-phenylazo[*h*]chromen-4-one) to inhibitor concentration of 30 μM resulted in small but pronounced changes in derivative spectra (decrease in the maximum amplitude, downshift of the spectral maximum at about 293 nm) corresponding to the exposure of tryptophans towards the solvent. Further addition of the inhibitor led to a decrease of the exposure of these aromatic side-chains. Similar behaviour was also observed in this work for other cytochromes P-450 (2B4 and 11A1). The fourth derivative of absorption spectra was also used to examine the stability of the enzyme both in the presence and absence of α-naphthoflavone, with increasing pressure (up to 400 MPa) and temperature (up to 35 °C) as pertubing factors. The results show that cytochrome P-450 1A2 has a stable conformation as all the conformational changes observed were (spectrally) fully reversible.
**Key words:** Cytochrome P-450; CYP1A2; α-Naphthoflavone; Conformation; Derivative spectra.

Cytochromes P-450 (P-450) are enzymes known to hydroxylate a variety of non-polar substrates of both endogenous and exogenous origin. Individual P-450 subfamilies differ in their substrate specificity which is determined by the structure of the respective active sites. Typical substrates of P-450 of the 1A subfamily are aromatic structures including polycyclic hydrocarbons, amines and other compounds which are prototypic carcinogens[1–3]. This is why there is in the literature a considerable focus on the structure and properties on both P-450s of this subfamily, namely, on the hepatic 1A2 as well as on the extrahepatic 1A1 isoform.

Because no liver microsomal cytochrome P-450 has been crystallized so far, there are numerous studies in the literature trying to get an insight into the structure of this

integral membrane proteins by comparison with the soluble bacterial P-450s which were crystallized and the three-dimensional structure of which is known[4–7]. These comparisons gave a valuable estimate of the overall shape of the protein molecules and of the putative amino acid composition of their active site. Spectroscopic methods are able to implement these pieces of information by data on subtle conformational changes taking place due to interactions with substrates and inhibitors, and due to the effects of temperature or pressure.

Direct observation of effects of conformational changes of proteins on the UV absorption spectra is difficult as there are many contributing bands present in the most important region between 260 and 310 nm. To enhance the resolution, the second[8,9] and fourth[10–12] derivative spectroscopies are used to monitor conformational changes involving the aromatic amino acid residues. In P-450s, second derivative spectra in the "aromatic" region were used to study for instance the accessibility of P-450cam (CYP101) tyrosine side chains to solvent[13] and to evaluate the differences between rat P-450b and P-450e (CYP2B1 and B2) in substrate-active site interactions[14]. As has been shown for model compounds[11], both the position and amplitude of the derivative spectra reflect the relative permittivity of the solvent. Hence, for proteins, the decrease in amplitude and blue shift of spectral maxima indicate an increase in accessibility of aromatic residues[11].

The P-450 1A2 is relatively rich in both the amino acid residues in question as it possesses nine tryptophans and eight tyrosines in the molecule. In this work, the stability of conformation of P-450 1A2 (CYP1A2) enzyme is studied under different conditions: (i) at different concentrations of its specific inhibitor – α-naphthoflavone, (ii) under high pressure (up to 400 MPa) and (iii) at different temperatures.

## EXPERIMENTAL

### Materials

The enzyme, human recombinant P-450 1A2, has been prepared from transformed *E. coli* cell membranes according to published procedure[15]. The transformed *E. coli* cells were a generous gift of Dr F. P. Guengerich of Vanderbilt University, Nashville, U.S.A. The buffer used was 0.3 M potassium phosphate with 0.2 mM EDTA and 20% glycerol (pH 7.4). The P-450 concentration was the same in all experiments (2.5 μM). The chemicals were of the reagent grade quality and were purchased from Sigma (St. Louis, U.S.A.).

### Methods

UV and VIS absorption spectra were taken with a Cary 3E (Varian) and with a Specord M40 (Zeiss, Jena) spectrophotometers equipped with thermostated cell holders, under conditions similar to those in ref.[11]. To study the effect of other molecules, the spectra were taken as the difference spectra, *i.e.*, by placing the protein and the interacting substance into the sample cuvette and the solution of the interacting compound of the same concentration into the reference cuvette to substract its spectrum. Effects of temperature were examined after short period (about 10 min) to let the sample to equili-

brate. The experiments with samples exposed to high pressure were realized using a thermostated (25 °C) high pressure cell[11] placed in the sample compartment of the Cary 3E. The fourth derivatives were obtained by numerical derivation based on the substractions of the spectra shifted by a defined Δλ value (to visualize the changes in the polarity of the tryptophan and tyrosine moieties, 1.6 and 2.6 nm windows were used, respectively) as recommended[11] using a simple Sigma Plot (Jandel Scientific) based procedure[11,16]. The spectral changes were expressed as maximum amplitude (see Fig. 1) *vs* the respective variable (pressure, temperature and ligand concentration).

## RESULTS AND DISCUSSION

### *Interaction of P-450 1A2 with α-Naphthoflavone*

α-Naphthoflavone is known to bind to P-450s of the 1A subfamily (1A1 and 1A2) as it is a specific inhibitor of their enzymatic activities[1–3] ($K_i$ = 10 μM for 1A2, see ref.[3]). However, the nature of this interaction has been studied only recently showing a competitive character of the process[3,17].

The fourth derivative spectra of the P-450 1A2 (Fig. 1) exhibit a course typical of a protein rich in aromatic amino acids giving two distinct maxima (at about 290–293 and 283–286 nm) and two minima (294–297 and 288–290 nm). To visualize the changes in the derivative spectra, the maximum amplitude *vs* α-naphthoflavone concentration was plotted. For both wavelength shifts (derivation windows) used, the dependence is very similar, with a decrease in the amplitude at α-naphthoflavone concentration at about 30 μM and a slow rise at higher ligand concentrations (for a window of 2.6 nm, see Fig. 2). This effect could be interpreted as an increased accessibility[11] of aromatic amino acid residues followed by their shielding (or burying) again at higher concentrations of the interacting small molecule. Taking into account the dissociation constant of the α-naphthoflavone–P-450 1A2 complex, the breakpoint observed takes place at concentrations corresponding to the formation of the approximately equimolar complex. This interpretation is also in line with a change in the second structure-sensitive parameter

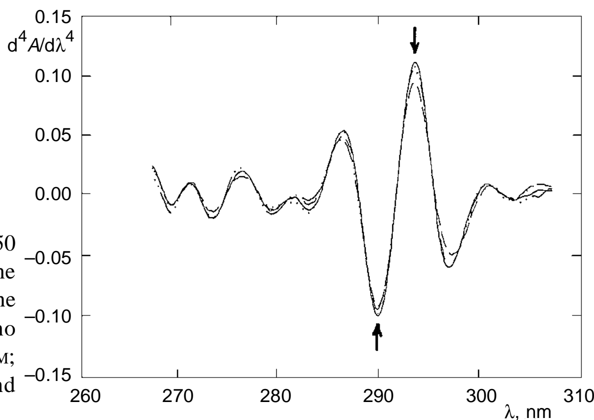


FIG. 1
Fourth derivative UV spectra of P-450 1A2 with different α-naphthoflavone concentration (the arrows denote the maximum amplitude). Full line, no inhibitor added; dotted line, 6 μM; dashed line, 30 μM; dashed and dotted line, 120 μM

of the fourth derivative spectra, in the position of the first maximum (at about 290–293 nm). The position of this maximum should primarily reflect properties of the tryptophan moiety[11]. In fact, there was a small but clear 0.3-nm blue shift of the maximum (Fig. 1) at concentration of about 30 μM indicating again better accessibility of tryptophan(s)[11].

For comparison, the same characteristics were followed for two well-known interactions: (i) binding of a typical substrate, benzphetamine, to cytochrome P-450 2B4 (LM2) and (ii) interaction of a specific substrate, 22(*R*)-hydroxycholesterol, with P-450 11A1 (P-450scc). In fact, the course of the respective curves were in both cases the same (Fig. 2) showing an increase of accessibility of aromatic residues followed by their burying after more substrate is added.

*Effect of Temperature*

The effect of temperature on the P-450 1A2 conformation was studied both in the absence and in the presence of α-naphthoflavone. The conformational changes observed are again small corresponding to better accessibility of the aromatic amino acid residues in question with increasing temperature (Fig. 3); moreover, they are well re-

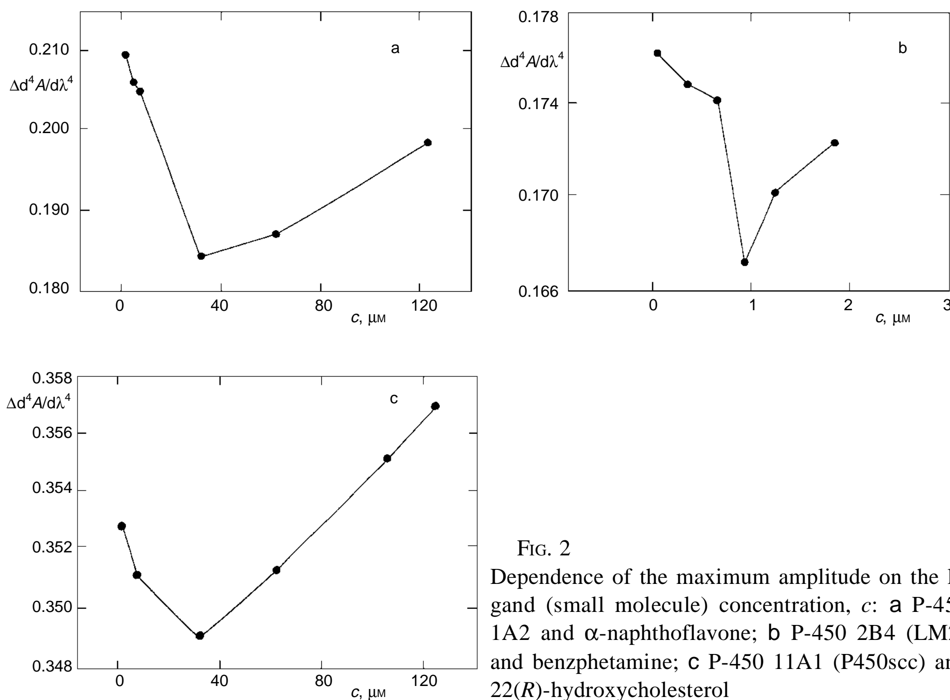


FIG. 2
Dependence of the maximum amplitude on the ligand (small molecule) concentration, *c*: a P-450 1A2 and α-naphthoflavone; b P-450 2B4 (LM2) and benzphetamine; c P-450 11A1 (P450scc) and 22(*R*)-hydroxycholesterol

versible (see Fig. 3 for changes of the maximum amplitude in the absence and in the presence of 30 μM α-naphthoflavone). The results show that the conformation of P-450 1A2 is highly stable in the temperature range studied.

*Effect of Pressure*

The pressure was applied on P-450 1A2 samples in the absence and in the presence of α-naphthoflavone in the same concentration as above. As a rule, proteins in aqueous media under pressure exhibit a decrease in the maximum amplitude of the fourth derivative spectra[18]. This was also the case with P-450 1A2 (Fig. 4). On the other hand, proteins are known to differ in their ability to withstand the effect of pressure and to

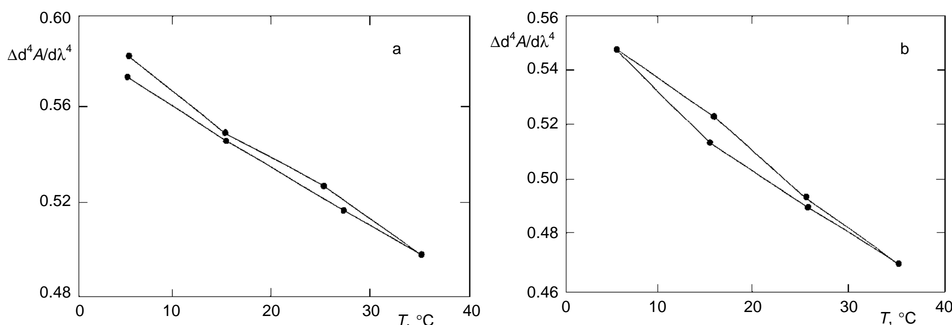


FIG. 3
Effect of temperature, *T*, on P-450 1A2 conformation. Dependence of the maximum amplitude in the fourth derivative spectra of P-450 1A2 on temperature taken in the absence (a) and in the presence (b) of the interacting molecules (30 μM α-naphthoflavone)

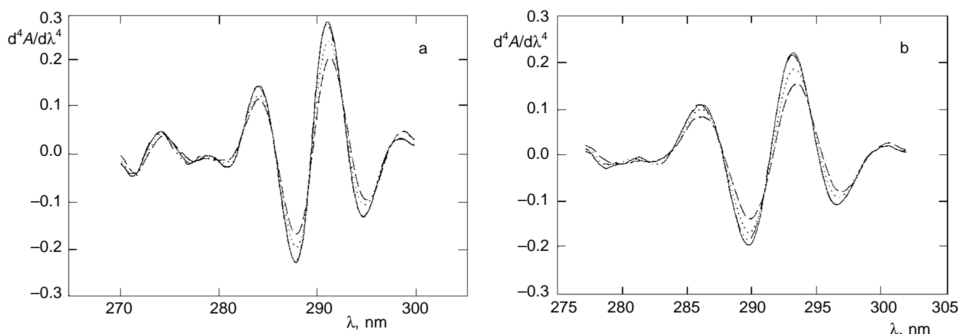


FIG. 4
Effect of pressure on the P-450 1A2 conformation. Fourth derivative spectra taken in the absence (a) and in the presence (b) of α-naphthoflavone. Full line, 1 MPa; dotted line, 150 MPa; dashed line, 300 MPa; dashed and dotted line, pressure released back to 1 MPa

return (once the pressure is released) to the initial state[11,18] (native form). The P-450s are not an exception: whereas P-450 2B4 (LM2) is rapidly converted to an inactive form of all P-450s, *i.e.* to the P-420, at pressure of about 100 MPa, P-450 2A5 is much more stable (up to 400 MPa, ref.[19]). The presence of polyols (*e.g.* glycerol) is known to slow down the denaturation process[11].

Conversion of P-450 1A2 to P-420 was followed by monitoring the formation of the absorption band at about 420 nm under increasing pressure in a sample of reduced P-450 in presence of carbon monoxide (Fig. 5). A weak shoulder at about 420 nm found at the beginning of the experiment may be either due to an incomplete reduction (a consequence of the presence of residues of oxidized species absorbing at about 417 nm) or to the traces of denaturated P-450 already present in the thawn sample. At pressures above 300 MPa, formation of the inactive P-420 is clearly seen (when the pressure is released, the P-420 content makes approximately 30% of the sample). Similar behaviour was also observed in the presence of 30 μM α-naphthoflavone.

The fourth derivative spectra were employed to monitor the conformational changes induced by the applied pressure (Fig. 4). As mentioned above, a decrease in the amplitude was observed similarly to the effects reported for other proteins[18]. Both in the absence and in the presence of the interacting molecule, the changes in the maximum amplitude were completely reversible in the range up to 300 MPa. On the other hand, in the presence of α-naphthoflavone, a considerable shift of the first maximum was observed (from 293.1 to 293.4 nm) at pressures reaching 300 MPa (Fig 4). The same values of the spectral shift were also reported *e.g.* for methanol dehydrogenase[18]. This finding can be interpreted as an indication of burying of aromatic amino acid residues (here, the tryptophan(s)), or, in other words, of lower accessibility of these residues at higher pressures. This conformational change is again completely reversible.

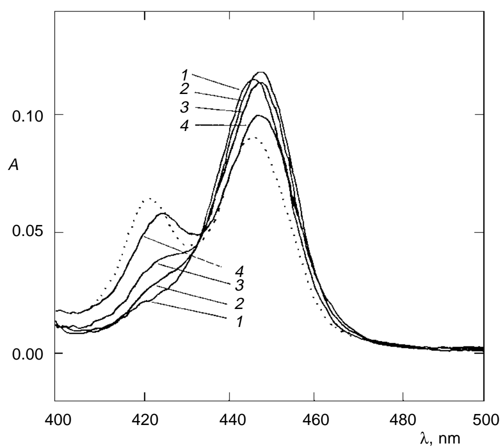


FIG. 5
Formation of inactive form (P-420). Reduced P-450 1A2 in the presence of carbon monoxide under pressure of 1 (*1*), 350 (*2*), 400 (*3*), 480 (*4*) and again 1 MPa (dotted line)

## *Conclusions*

The results presented here document a considerable stability of the P-450 1A2 enzyme structure under different conditions. When an interacting molecule (specific inhibitor) is present, the conformation of the enzyme changes only slightly (minor changes were observed for aromatic amino acid residues (tryptophans) in all the P-450s studied). Increasing temperature from 5 to 35 °C caused neither denaturation nor any substantial conformational changes. The pressure indeed causes denaturation; however, the 1A2 enzyme is much more stable than the P-450 2B4 (LM2) (widely used in the literature as a model system). In this respect, it compares well with another microsomal P-450, the CYP2A5 (ref.[19]). The conclusions reached here are based on interpretation of spectral properties of the aromatic amino acid residues in the protein. However, they may be indicative for the behaviour of the whole P-450 1A2 molecule as comparisons with models of the 1A2 enzyme[5,6] show that the tyrosines and tryptophans (eight and nine residues, respectively) are rather evenly distributed. Hence, the P-450 1A2 can be taken as an example of a membrane cytochrome P-450 with a relatively stable conformation.

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