ACCESSIBILITY OF TYROSINE RESIDUES IN CYTOCHROME P-450scc (CYP11A1)

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Dedicated to Dr Jan Fajkos on the occasion of his 75th birthday.

Cytochrome P-450scc (CYP11A1) is known to exist in various conformational states. To study the accessibility of tyrosine residues to the solvent, second and fourth derivatives of cytochrome absorption spectra in the ultraviolet region were used. The measurements were carried out in the temperature range –10 to 40 °C and at two pH values (6.8 and 7.4). Our results indicate that the tyrosine residues of this enzyme are less accessible at higher temperatures as well as at higher pH, that is at conditions where the conformational equilibrium shifts to low-spin Fe(III) form(s). In other words, a more compact structure is attributable to the low-spin Fe(III) form(s) of P-450scc.

Key words: Enzymes; Cytochromes P-450; CYP11A1; Derivative spectra; Steroid hormones biosynthesis.

Cytochromes P-450 (P-450s) of adrenal cortex are known to take part in biosynthesis of steroid hormones. The first and rate-limiting step in the synthesis of most steroid hormones is the cleavage of the cholesterol side chain leading to formation of pregnenolone[1,2]. This reaction takes place in the inner membrane of adrenal cortex mitochondria and is catalyzed by cytochrome P-450 11A1 (abbreviated CYP11A1, also P-450scc for "side chain cleavage")[3,4]. The physiological importance of this enzyme together with unusual properties based on its structure and presence of several forms has recently attracted much attention[5–9]. This mitochondrial cytochrome P-450 has been shown to exist in two conformational states, in complex equilibrium with their protonated forms and with low spin and high spin forms of the heme iron atom[7,8]. The experimental data collected suggest that the forms also differ in the amount of solvent entrapped in the protein structure.

Here, the absorption spectroscopy in the ultraviolet region can bring additional information as the position and amplitude of the derivative spectra were shown to reflect the changes in accessibility of aromatic amino acid residues. Both the second- (refs[10,11]) and fourth-derivative (refs[12–14]) spectra in the "aromatic" region, between 260 and 310 nm, were used to enhance the resolution of the absorption spectra as there are more contributing bands present in this region making analysis of the spectral curve difficult. Ragone *et al.*[10] introduced the parameter *r* which can be easily calculated as the ratio of the two amplitudes of the second-derivative spectrum (at about 283 to 287 nm, and 290 to 295 nm; see Fig. 1). They concluded that the value of the parameter *r* reflects the polarity of the medium in which tyrosyl residues are embedded (larger *r* values were found for more polar environment of tyrosine). The fourth derivatives were inspected by Lange *et al.*[13,14] who found that the decrease in the maximum amplitude of the spectrum and blue shift of the spectral maxima indicate an increase in accessibility of aromatic residues.

In P-450s, second-derivative spectra helped to evaluate the accessibility of tyrosines in bacterial soluble P-450cam (CYP101)[15] and the differences between rat isoforms P-450b and P-450e (CYP2B1 and CYP2B2) in substrate–active site interactions[16]. The fourth-derivative spectra were used recently to study the conformational stability of P-450 1A2 (ref.[17]).

In this work, the accessibility of tyrosine residues in the molecule of P-450scc at different temperatures is investigated by examining the second and fourth derivatives of absorption spectra. The results (at two pH values) are discussed together with data on the spin equilibrium at corresponding temperatures. In the second part of this study, the properties of the tyrosine residues are examined under an increasing pressure.

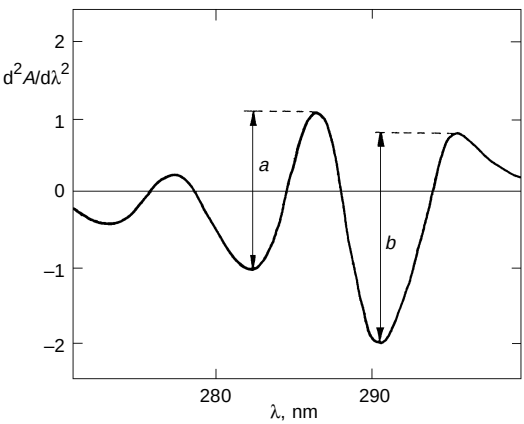

FIG. 1
A schematic picture of the second derivative of the absorption spectrum of a protein showing the definition of parameters *a* and *b*

EXPERIMENTAL

Materials

The enzyme, cytochrome P-450scc, was prepared from bovine adrenal cortex mitochondria according to Suhara[18]. Protein concentration, specific P-450 content determinations and controls in the course of the preparation were carried out as described previously[19]. The purified enzyme was kept in 50 mM phosphate buffer (pH 7.4) with 10% glycerol at –80 °C until used. Before spectrophotometric experiments at different temperatures, the glycerol content was increased to 30% and pH was adjusted to the desired value. Experiments under high pressure were done with the protein sample as it was prepared. Enzyme concentrations were 3 μmol l^{-1}. All chemicals used were of the highest commercial quality and were purchased from Sigma (St. Louis, U.S.A.).

Methods

The second derivatives of the absorption spectra were recorded directly with a Specord M40 (Carl Zeiss Jena) spectrophotometer equipped with a Data Handling accessory. The samples were placed in a thermostated cell holder in a home-built housing unit with silica gel to prevent freezing of water vapour at temperatures below 0 °C. From the spectra, the parameter r (ref.[10]) was calculated as the ratio of $r = a/b$ where a is the amplitude of the first (at about 283 to 287 nm) and b of the second (290 to 295 nm) extreme as shown in Fig. 1. The fourth-derivative spectra were obtained from the data recorded using a Cary 3E (Varian) spectrophotometer equipped with a thermostated high-pressure cell[13] placed in the sample compartment of the Cary 3E. The fourth derivatives were calculated by numerical derivation based on the subtractions of the spectra shifted by a defined $\Delta\lambda$ value (to visualize the changes in the polarity of the tyrosine moiety, a 2.6 nm window was used as recommended[13]) using a simple Sigma Plot (Jandel Scientific) based procedure described previously[13,17]. The spin state of heme iron was determined from absorbances at 420 and 390 nm and expressed as the spin equilibrium constant K (defined as the ratio of concentrations of the high-spin and low-spin forms of P-450, K = [high-spin]/[low-spin]) (ref.[6]).

RESULTS AND DISCUSSION

Temperature changes in the exposure of the tyrosine residues expressed as the ratio r for two different pH values of the buffer solution used are shown in Fig. 2. The r values increase with decreasing temperature, thus indicate hiding of the tyrosine residues during the increase of temperature. The course of the changes is more or less uniform; to decide on the possible stepwise course of the conformational changes, a more thorough study would be necessary. The dependence preserves its character also at higher pH (7.4). The results indicate that at higher temperatures the interior of the enzyme (or, at least the "average environment" of the 20 tyrosine residues[20] of P-450scc) becomes less accessible for the solvent. Similar hiding of tyrosine residues in P450scc (at pH 7.4) with growing temperature has been observed in near-UV CD spectra[21].

In the same figure (Fig. 2), the values of the spin equilibrium constant K at pH 6.8 are plotted against temperature, indicating increase of low-spin content with temperature (similar change in P-450scc heme-iron spin state was previously observed[6] also for aqueous solution and for 35% ethylene glycol in pH range 6.8–8.4). When the data on

the spin state are taken into account, it can be concluded that in the low-spin form(s) prevailing at higher temperatures the tyrosines are less accessible. This fact is in line with the view presented by Bancel *et al.*[8] that the low-spin P-450scc possesses a more compact structure.

The results for two different pH values show that at lower pH, the structure in the vicinity of the tyrosine moieties is more open to solvent molecules as the *r* values are higher indicating better accessibility of the tyrosine residues. Using the fourth-derivative spectra, Bancel *et al.*[8] also found an increase of solvation of aromatic amino acids at lower pH (6.8) compared with data for pH 7.4. Because the increase of pH shifts the

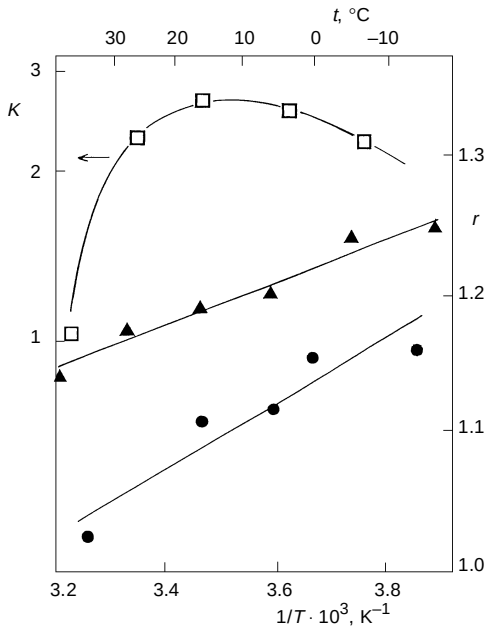

FIG. 2
Dependence of the ratio $r = a/b$ on the temperature at two pH values (▲ pH 6.8; ● pH 7.4). In the same graph, the dependence of the spin equilibrium constant K on the temperature at pH 6.8 is shown (❒). For explanation of symbols, see the text

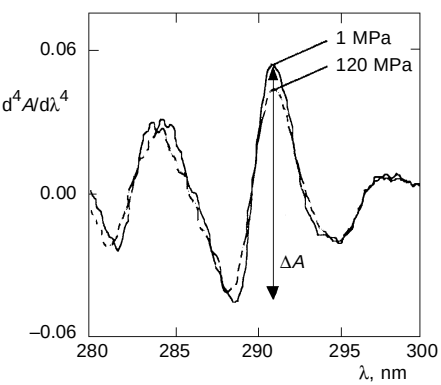

FIG. 3
Fourth derivative of the absorption spectra of P-450scc, pH 7.4 recorded at hydrostatic pressure of 1 MPa (full line) and 120 MPa (dotted line)

spin state equilibria towards low spin[21], this findings again support the picture of the compact low-spin form of P-450scc.

The changes of the accessibility of tyrosine with the increased pressure were examined by fourth-derivative spectroscopy. Tyrosine contributes to the spectrum in the region between 280 and 290 nm. The position of the absorbance maximum at about 285 nm should, in principle, reflect the changes in the polarity of the environment of the tyrosine residues[13] however, the signal in this region cannot be easily resolved apparently due to high number of tyrosine moieties present (twenty; ref.[20]). On the other hand, the tryptophan residues are apparently more accessible to the solvent under increased pressure (a decrease in the ΔA, see Fig. 3, is known to reflect a better accessibility of tryptophan residues[13]).

In conclusion, the results of this study document the increase of accessibility of aromatic amino acids in P-450scc with lowering of pH and temperature and at high hydrostatic pressure (120 MPa), indicating existence of a less compact structure of the enzyme under these conditions.

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