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High pressure induced inactivation of ferrous cytochrome P-450 LM2 (IIB4) CO complex: Evidence for the presence of two conformers in the oligomer

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The effect of high pressure on the spectral properties of cytochrome P-450 LM2(Fe^{2+})-CO complex was studied. The application of high pressure was shown to induce the conversion of cytochrome P-450 to P-420. In the solution when P-450 was oligomeric only about 65% of the total converted to P-420. The remaining portion of cytochrome P-450 was stable at pressures up to 6 kbar. When P-450 was incorporated into membranes or when it was succinylated, the proportion of the pressure sensitive fraction was slightly higher (about 75%). Dissocation of P-450 oligomers into monomers was made by addition of 0.2% Triton N-101. Monomers were the most sensitive to pressure; they could be completely converted to P-420. These results have been interpreted as evidence for the existence of two different conformers of P-450 LM2, which differ in pressure stability. Splitting between these two states appears to be a result of the oligomeric organization of cytochrome P-450 in solution and in the membrane.

The structure of microsomal cytochrome P-450 and the organization of the microsomal monooxigenase has been extensively studied by numerous groups. However, some general questions still remain. Of these, one of the most intriguing concerns the biphasic reduction kinetics of microsomal cytochrome P-450. At least for several microsomal cytochromes P-450, the fast phase of reduction corresponds to the reduction of the high-spin form of the hemoprotein (1,2). Thus, at the end of the fast phase the high-spin fraction of cytochrome P-450 vanishes. The spin transitions of cytochrome P-450 are extremely fast processes (3,4); one might expect that the kinetic curves for the reduction of high-spin and low-spin hemoprotein should be identical. To date, we have no explanation for the discrepance between expectation and experiment. It appears, that microsomal cytochrome P-450 is split between two conformational states; these

Abbreviations: EDTA, ethylendiaminetetraacetic acid; HEPES, N-hydroxyethylpiperazine-N-ethane-sulfonic acid; LM2, rabbit liver microsomal cytochrome P-450 LM2 (IIB4).

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states have different spin equilibria and different mechanisms of interaction with NADPH-cytochrome P-450 reductase (4,5). The idea that there is splitting of cytochrome P-450 into different conformers was also established earlier based on the studies of the kinetics of cytochrome P-450 reduction by dithionite (6) and cytochrome P-450 - carbon monoxide complex recombination kinetics (7).

High pressure has been successfully used for the studies of conformational dynamics of bacterial cytochromes P-450cam and P-450lin (9-13). In these cytochromes an application of a high pressure results in a dissociation of cytochrome-substrate complex which is accompanied by a shift of the spin equilibrium (10,11). At the pressures, higher than 2 kbar, P-450cam is converted into the inactive P-420 state. This transition was also detected in P-450cam(Fe^{2+})-CO complex (11).

The present study was undertaken in attempt to use high pressure-induced changes in microsomal cytochrome P-450 LM2(Fe^{2+})-CO complex to examine the conformational splitting of this hemoprotein in the oligomers and monomers in solution as well as in the membrane-bound state.

Materials and methods

Materials - Electrophoreticaly homogenous cytochrome P-450 LM2 (IIB4) was purified from the liver of phenobarbital-treated rabbits (14). Cytochrome P-450 preparations were stored in 0.1M potassium phosphate buffer containing 1mM EDTA, 1mM dithiothrietol and 20% glycerol at -70 °C. The preparations used contain no more than 15% of P-420 state of the hemoprotein. Succinylation of cytochrome P-450 LM2 was made as described earlier (15). L-α-phosphatidylcholine from fresh egg yolk (type XI-E) and l-α-phosphatidylethanolamine from bovine liver, HEPES, EDTA and D,L-dithiotreithol were obtained from Sigma Chemicals. Triton N-101 from Fluka and sodium dithionite from Merk were used. Carbon monoxide was a product of the reaction of the sulfuric and formic acids. It was bubbled through 0.2M solution of sodium dithionite in 0.5M NaOH to remove the traces of oxygen.

Preparation of the proteoliposomes. To produce the proteoliposomes Sephadex LH-20 gel filtration technique (16) was used. Chloroform solutions of L-α-phosphatidylcholine and L-α-phosphatidylethanolamine were mixed and evaporated under the flow of argon to produce 4 mg of 9:1 (w./w.) mixture of the phospholipids. Stock solution of the purified LM2 (10 nmoles of hemoprotein) was added to the pellet. 2 ml of O.1 M Na-HEPES buffer, pH 7.4, containing 0.4% Triton N-101, 1 mM EDTA and 1 mM dithiotriethol were added to the sample. It was vigorously mixed with a Vortex mixer to produce a clear, slightly opalescent suspension and applied on 1.8x30 cm column of Sephadex LH-20 (Pharmacia, Sweden), equilibrated with 0.1 M Na-HEPES buffer, containing 1 mM EDTA and 1 mM dithiotriethol (HEPES-buffer) and eluted with the same buffer. Cytochrome P-450-containing proteoliposomes were found in the void volume of the column. The release of the hemoprotein and the detergent were controlled following the optical density at 420 nm and 270 nm respectively. Cytochrome P-450-contained fractions were combined and centrifuged for 90 min. at 105 000 g . The pellet of the proteoliposomes was resuspended in a small volume (0.2 - 0.3 ml) of HEPES buffer, containing 20% glycerol and stored at -12 °C before used.

High-Pressure system. The optical pressure system, capable of generating a pressure of 6200 bar, has been described previously (17). The bomb was fitted directly into the sample compartment of Cary-219 spectrophotometer. The high-pressure system was fully computer-controlled. High-pressure system and spectrophotometer control, data acquisition and spectra decomposition analysis were done by IBM-AT computer equipped with a special software package, SpectraLab, which should be described in details elsewhere.

Experimental procedure. All the pressure experiments were made in 0.1 M Na-HEPES buffer, pH 7.4, containing 1 mM EDTA and 1 mM dithiotriethol at 4 °C. It was shown by the control

measurements that pH of this buffer decreases by 0.06 unit per kilobar. The samples of ferrous cytochrome P-450-CO complex were prepared by the following procedure. 12.5 mM solution of sodium dithionite in HEPES-buffer was gently bubbled with CO for 1 min. Stock solution of cytochrome P-450 or the suspension of proteoliposomes was added to the buffer thermostated at 4 °C to yield the concentration of P-450 in the range of 1.5-4 nmol/ml for the soluble cytochrome P-450 or 0.6-1.0 nmol/ml for the proteoliposomal suspension. The sample was placed into the measuring cell and transferred into the bomb, thermostated at 4 °C.

Processing of the spectra. To compensate the effect of the turbidity, which appears at the pressures higher than 2.5 kbar, the method of the algebraic baseline correction (18) with a third-order polynomial was used. The corrections of the spectra on the compression of water were done automatically by the data acquisition program using the known coefficients of the compression (19). To follow qualitatively the changes in cytochrome P-450 and P-420 concentrations, the computer program, based on the principles of factor analysis (20) was developed. The spectra of pure cytochrome P-450 LM2 and pure P-420-state of this hemoprotein were used as standards. Cytochrome P-420 was produced by an incubation of LM2 in the presence of 1.6 M NaSCN (21). To normalize these spectra to 1 μM concentration of hemoprotein the extinction coefficients, given by Imai and Sato (21) were used. To fit the data the equation for the dependence of equilibrium constant on pressure

$$K_{eq}=\exp[(p_{1/2}-p)\Delta V°/RT] \quad (1)$$

was used. Here K_{eq} - apparent equilibrium constant for the conversion of P-450 to P-420 at pressure p, $p_{1/2}$ - the pressure at which $K_{eq}=1$ ("half pressure" of the conversion), $\Delta V°$ - molar volume change, associated with the conversion at atmospheric pressure. This equation was transformed to yield the following relationship:

$$\Delta[\text{P-420}]_p/[\text{P-450}]_o=A_o+F_c\cdot(\exp[(p_{1/2}-p)\Delta V°/RT]+1)^{-1} \quad (2)$$

$\Delta[\text{P-420}]_p$ - pressure induced changes in cytochrome P-420 concentration, $[\text{P-450}]_o$ - total concentration of cytochromes P-450 and P-420 in the sample, F_c - the fraction of cytochrome P-450 exposed to the conversion, A_o - the constant, which reflects the position of apparent equilibrium at the room pressure. Fitting of concentration curves to find F_c, A_o, $p_{1/2}$ and $\Delta V°$ was made using the computer program, based on Marquardt (22) and Neilder-Mead (23) optimization algorithms. The curves for [P-420] increase and [P-450] decrease were fitted separately and the results of both fittings were averaged.

Results and discussion

It was found, that in all the systems studied an application of high pressure converts cytochrome P-450 LM2(Fe^{2+})-CO complex into P-420(Fe^{2+})-CO (Fig. 1). For succinylated LM2 and the cytochrome in the presence of detergent this process was accompanied by a small decrease of the total concentration of P-450 and P-420 at pressures higher than 3 kbar. Such decrease probably results from loss of heme by cytochrome P-450 at high pressures. Additional spectral changes were the result of some red shift and broadening of P-420(Fe^{2+})-CO peak at high pressures. To take into consideration these changes, the spectrum of cytochrome P-420, recorded at 2.5 kbar were used as a standard for the spectra decomposition. This standard was used along with the 1 bar spectra of cytochrome P-450(Fe^{2+})-CO and P-420(Fe^{2+})-CO.

The changes in cytochrome P-450 and P-420 concentrations with increasing pressure are shown in fig. 2. In the oligomeric state pressure-induced LM2 inactivation is characterized by $P_{1/2}=3.0\pm0.5$ kbar and $\Delta V°=50\pm8$ ml/mole ($\Delta V°$ values given in this paper corresponds to the transition of one mole of LM2 monomer). Only 65 ± 9 % of oligomeric LM2 is exposed to the in-

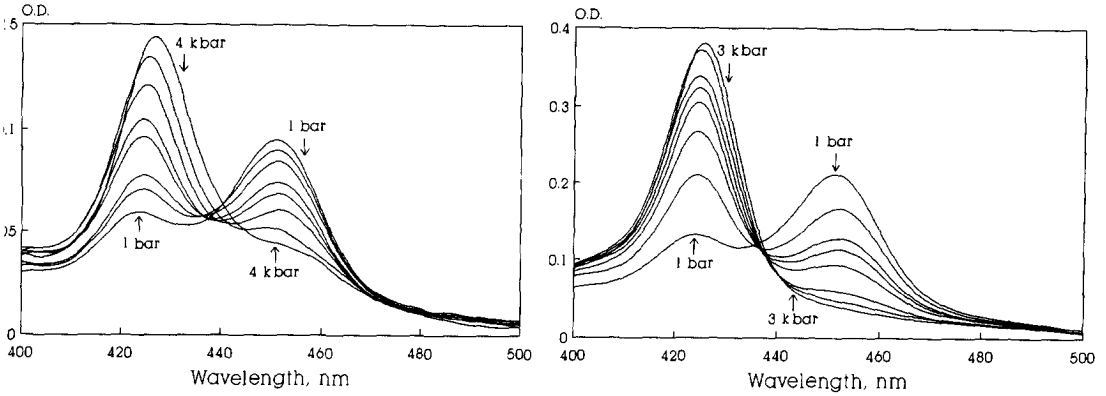

Fig. 1. Spectral changes in ferrous P-450 LM2 - CO complex due to increasing pressure. Spectra were recorded at the conditions described in "Materials and Methods". Optical path of the high-pressure cell was 4.8 mm. *Left:* In the solution of oligomeric P-450 LM2 (1.7 nmol/ml). Spectra recorded at 1bar and 1.6, 2.0, 2.4, 2.6, 3.0, 3.4, 4.0 kbar are shown. *Right:* In the solution of monomeric P-450 LM2 (3.8 nmol/ml) in the presence of 0.2% Triton N-101. Spectra recorded at 1bar and 1.2, 1.4, 1.5, 1.6, 1.8, 2.0, 3.0 kbar are shown.

activation process. The rest of the hemoprotein is more stable and cannot be converted into P-420-state at the pressures up to 6 kbar.

Non-ionic detergents like Triton N-101 or Emulgen-913 at concentrations 0.2% and higher were shown to dissociate cytochrome P-450 hexamers (6,24). In our experiments 0.2% Triton N-101 was used. In the presence of this detergent 95±10 % of cytochrome P-450 LM2 is exposed to pressure-iduced inactivation with $P_{1/2}$=1.3±0.2 kbar and $\Delta V°$=104±37 ml/mole. Thus, solubilization of the oligomers by Triton N-101 leads to suppression of the divergence of LM2 into two fractions with different pressure sensitivity. In the presence of this detergent cytochrome P-450 LM2 becomes more sensitive to pressure. Monomeres of LM2 are comparable in the pressure-related properties with bacterial cytochrome P-450cam. Increase of $\Delta V°$ of P-450 LM2 with the monomerization shows that the difference between oligomeric LM2 and $P\text{-}450_{cam}$ in the pressure stability results from the steric limitations of cytochrome P-450 LM2 conformational mobility in the oligomers.

Succinylation of cytochrome P-450 changes the surface charge of the molecule, disturbing protein-protein interactions in the oligomers. We have found that it leads to some increase of the pressure-sensitive fraction of cytochrome. In these conditions about 78±20 % of hemoprotein is exposed to the inactivation with $P_{1/2}$=2.5±0.5 kbar and $\Delta V°$=54±26 ml/mole. Solubilization of succinylated LM2 oligomers by 0.2 % Triton N-101 has the same effect, as for unmodified protein.

The pressure-related properties of LM2 in the proteoliposomes are close to those of succinylated cytochrome. In this case 74±16 % of the total content of cytochrome is exposed to pressure-dependent inactivation with $P_{1/2}$=2.2±0.2 kbar and $\Delta V°$=77±23 ml/mole. Thus, the divergence of P-450 into two fractions, differing in pressure stability, takes place not only in the solution, but also in the membrane. This fact agrees with the known evidences, that cytochrome P-450 LM2 forms oligomers (hexamers) not only in solution (24), but also in the membrane (25-29).

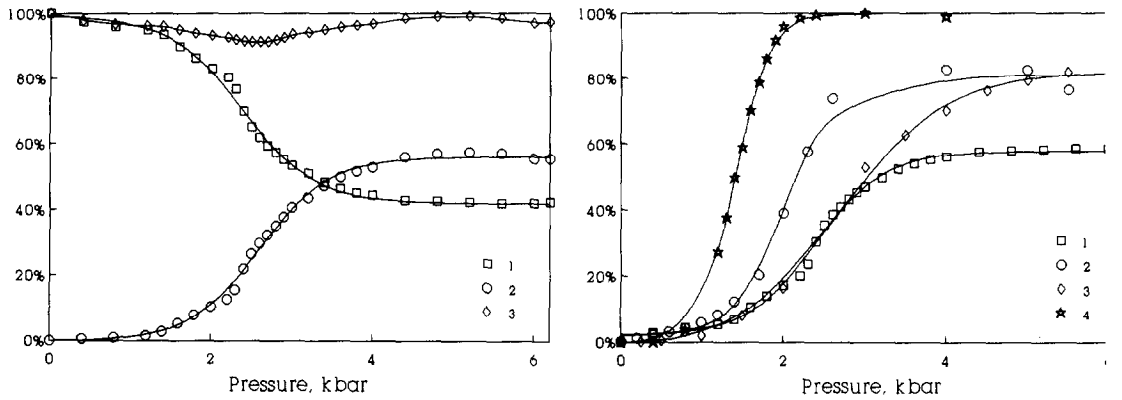

Fig. 2. Pressure-induced conversion of P-450 LM2 to P-420. Contents of P-450 and pressure-generated P-420 in the sample were expressed as percent ratio on the initial concentration of P-450. The experimental data points are shown along with the curves resulting from the data fitting to eq. (2). *Left:* Changes in P-450 (1) and P-420 (2) concentrations and the sum of both curves (3) for the solution of oligomeric LM2. *Right:* Changes in cytochrome P-420 concentrations measured in various systems: in the solution of unmodified oligomeric LM2 (1), solution of succinylated LM2 (2), proteoliposomes with incorporated LM2 (3) and monomeric LM2 in the presence of 0.2% Triton N-101 (4).

The data shown led us to conclude that LM2 in the oligomers (hexamers), and, likely, in the proteoliposomal membranes, is split between two conformers. These conformers have different pressure stability and there is no interconversion between them within the time of experiment. Concerning the hexamers in solution we can deduce that in each hexamer particle there are 4 molecules (67%) which are more flexible and, probably, more exposed to the water surrounding than the other 2. The latter two molecules are more rigid and pressure-resistent.

It was found erlier that the kinetics of dithionite-dependent reduction of cytochrome P-450 LM2 is biphasic in all the systems, but not in monomers of the hemoprotein (6,15). The fraction of the fast phase in this process was about 65% and this walue was independent on the temperature and the presence or absence of the substrate. Solubilization of the oligomers and membranes converts this kinetic into monophasic one (6). These data are in a good agreement with the former conclusion.

The present evidence on the splitting of cytochrome P-450 pool between two conformers in the oligomers and in the membrane appears to be an important finding for the understanding of molecular organization of microsomal monooxigenase system.

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