CROATICA CHEMICA ACTA CCACAA 48 (1) 69—86 (1976)

CCA-918

YU ISSN 0011-1643
577.15
Original Scientific Paper

Hepatic Cytochrome P-450. A Proton Magnetic Relaxation Study of Microsomal, Solubilized and Partially Reconstituted Enzyme System

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Received September 25, 1975

The longitudinal proton magnetic relaxation times, T_1, were measured from —5 to 40 °C for microsomal, solubilized and reconstituted cytochrome P-450 obtained from phenobarbital-induced rat livers. The paramagnetic contribution to the rates was derived by subtraction of the rates measured on dithionite-CO-reduced samples. The same values were obtained for microsomal P-450 on reduction with NADPH. PMR titration by KCN yielded a dissociation constant of about 30 mM. This is three orders of magnitude larger than for metmyoglobin. It is concluded that the measured PMR rates are most likely due to the P-450 (and P-420) haem-iron while the 30% non-haem iron found in both the microsomal and solubilized P-450 is ineffective for the PMR rates. These rates increase several times on isotopic dilution (D_2O for H_2O) with the microsomes and diminish for the solubilized samples. Microsomes show 17% residual, encaged, H_2O. Most of their paramagnetic PMR rate is due to the paramagnetic iron located on the outside of microsomes. This is demonstrated by measurements with deuterated samples to which 19% H_2O had been added. Hence, the solubilized P-450 is homogeneous regarding PMR, but the microsomes are not.

The paramagnetic molar relaxation rates are very high (10^3 to 2×10^3 s^{-1} M^{-1}) for the predominately low-spin iron in these samples. If the only relaxation mechanism is the magnetic dipole-dipole interaction of electron and nuclear spins, then the correlation time ought to be of the order of 10^{-10} s. Lack of knowledge of the actual relaxation mechanism prevents quantitative evaluation of the results. The comparison of relaxation rates measured by water protons, or (in deuterated solution) by aliphatic protons of deuterated glycerol indicates a very accessible haem-iron. The Arrhenius plots show clearly two temperature regions with the kink between 13 and 20 °C, both for the original and the CO-reduced samples. The molar paramagnetic rates derived as differences from the former pairs of data (original v. CO) have a PMR discontinuity around 18 °C. The PMR rates for the microsomes are twice as large as those for the solubilized P-450. When the latter is combined with the solubilized NADPH-dependent reductase, the PMR rates almost coincide with those of the original microsomes. It thus appears that the missing phospholipid in the reconstituted sample is not determining the conformation around the haem-iron. Rather, interactions of first-neighbour protein molecules separated possibly by

one lipid molecule bring about a functional (?) conformation sensitive to structural changes in the membrane around the »transition« temperature.

INTRODUCTION

Cytochrome P-450, a mixed-function oxygenase, is the key-constituent of an enzyme system for hydroxylation or demethylation of drugs in organisms[1-4]. Its role is to fix both the substrate and the oxygen molecule in a proper spatial arrangement for a two-step electron transfer with subsequent attachment of one oxygen atom to the substrate.

The very first event in this enzymatic cycle is considered to be the binding of the substrate to P-450 while the haem-iron of the latter is in the oxidized, ferric state. The enzymes from microorganisms are soluble in their native form. Those from the adrenal cortex and liver are membrane-bound. From the microsomes formed by the endoplasmic reticulum of hepatocytes partially purified cytochrome P-450 can be obtained. Hence, the elucidation of the molecular mechanism of this enzyme requires information on the structure of the immediate haem-environment both regarding the different structural levels in which cyt P-450 has been found to function, and the detailed mode of interaction with a wide variety of substrates.

Most of our knowledge about the structure/function relationship for P-450 has been obtained from equilibrium and kinetic binding-data using mainly difference spectrophotometry and electron spin resonance (ESR). These experimental techniques are particularly suited to determine the spin-state of the haem-iron and its changes under allosteric or direct influence of bound substrates. These questions require a structural characterization of the haem-iron environment. This has been done in a certain way with an ESR spin-label covalently bound to the haem-iron[5], but nuclear magnetic resonance (NMR) could be even more appropriate for this purpose[6]. Though perhaps most useful, its high-resolution mode may present difficulties with different structural states of P-450.

The proton magnetic *relaxation* (PMR) method was also found to yield useful structural and dynamic information for *high-spin* ferric iron in various haemoglobins and myoglobins[7]. As the measured effect depends on the electron--relaxation rate(s) of the haem-iron and not on rotational tumbling rate of the whole complex bearing the iron-ion, the haem-iron in P-450 could, in principle, be used as a non-perturbing natural structure-probe. Such an approach has already been attempted for the soluble bacterial P-450 whose haem-iron could be made either high- or low-spin[4,8]. The purpose of our work is to define clearly the problems in the evaluation of PMR results obtained with predominately *low*-spin haem-iron of rat-liver cytochrome P-450 and to relate the PMR data to the different states in which cyt P-450 may be isolated.

EXPERIMENTAL

Preparation of Microsomes

Male Wistar rats (120—150 g) were pretreated for 3 days with sodium phenobarbital (40 mg/kg body weight) dissolved in physiological saline. Twenty four hours before being killed the animals were deprived of food, while drinking *ad libitum*. After decapitation and bleeding the livers were removed and stored in ice-cold 0.1 M phosphate buffer, pH 7.4, containing 2 mM EDTA. The haemoglobin being removed by several washings the livers were cut and homogenized (with a Potter-

-Elvehjem) taking 3 parts of phosphate buffer per one part of liver. The suspension was centrifuged for 10 min at 3000 g, and the supernatant fluid was recentrifuged for 20 min at 10000 g. The post-mitochondrial supernatant was then centrifuged for one hour at 105000 g, and the pellets were washed twice with Krebs-Ringer-phosphate solution, pH 7.4[9], and centrifuged again. The microsomal pellets were resuspended in a 0.1 M phosphate and 0.25 M saccharose solution, pH 7.4. The protein concentration was about 40 mg/ml. After cooling in liquid nitrogen the microsomes were stored without deterioration at about —20 °C. All manipulations were performed up to 4 °C.

Solubilization of Microsomes and Partial Purification of Cytochrome P-450

Solubilizations were carried out as described by Lu et al.[10]. Microsomes (about 2 g) were thawed and diluted to a protein concentration of about 16 mg per ml in 0.1 M TRIS buffer, pH 7.4, containing 20% (v/v) glycerol, and 1 mM dithiothreitol (all final concentrations). The microsomal suspension was sonicated with an Ultrasonic Desintegrator (Branson B-12, Branson Sonic Power Comp., Danbury, USA) at full output (90 W) for ten 20 s intervals while the temperature of the suspension was kept below 5 °C. In the following ammonium sulphate fractionation steps precipitates were collected between 40 and 50% saturation, rather than between 43 and 50% sutaration as reported by Lu et al.[10].

Dialysis was performed in 0.1 M potassium phosphate buffer, pH 7.4, with 20% (v/v) glycerol, and 0.1 mM dithiothreitol. The clear supernatant obtained after 105000 g centrifugation for one hour usually contained 50 μM cytochrome P-450, 15 μM cytochrome P-420, and 6 μM cytochrome b_5 at a protein concentration of about 15 mg per ml. Higher concentrations up to 700 μM cytochrome P-450 were obtained by ultrafiltration using Amicon membranes XM-50.

Isolation of P-450-reductase and its Reconstitution with Solubilized Cytochrome P-450

The NADPH-dependent-cytochrome P-450-reductase of rat-liver microsomes was prepared according to Lu *et al*[10,11] with slight modifications. The isolated and solubilized reductase was frozen in liquid nitrogen and stored at —12 °C.

The activity of the preparation was determined by the method of Ernster *et al.*[12] and found to amount to 18460 U/ml or 296 U/mg protein (expressed as nanomoles cytochrome c reduced per min).

Several preparations were collected and subsequently concentrated by ultrafiltration. The final preparation of the reductase contained about 62 mg/ml protein.

Reconstitution of the hydroxylating system was performed by mixing the reductase with cytochrome P-450 in defined proportions. The volume of 0.7 ml reductase corresponding to 12920 U was added to 0.7 ml cytochrome P-450 (eq. to 438 nanomoles cyt c reduced per min). Thus, the reconstituted system contained about 30 U of reductase per nanomole cytochrome P-450.

The activity of the reconstituted system was proved by determination of the demethylase activity[13] before relaxation measurements. The amount of 14 nanomoles formaldehyde per nanomole cytochrome P-450 were produced at a final concentration of 10 millimole of aminophenazone.

Chemicals

Sodium cholate supplied by FERAK was used without further purification. Methphenetamine hydrochloride (Spasman®) was acquired from Arzneimittelwerk Dresden, GDR. Aniline was used only after distillation. All other reagents were analytical grade products.

D_2O was 99.8% produce by PROCHEM, London, England.

Analytical Methods

The protein content was determined by the method of Lowry *et al.*[14] with ovalbumin as standard.

The concentrations of cytochrome P-450 and cytochrome P-420 were determined from the CO-difference spectra using the absorption coeficients of 91 mM^{-1} cm^{-1} between 450 and 490 nm[15], after correcting for the negative contribution of the cytochrome

P-450 in the difference spectra at 420 nm with $\varepsilon = -41$ mM^{-1} cm^{-1} [16]. The cytochrome b_5 content was assayed from the difference spectra of NADH-reduced and oxidized forms by $\varepsilon = 112$ mM^{-1} cm^{-1} for the increment between 424 and 500 nm[17].

The total haem was derived from pyridine haemochrome spectra as described by Schenkman *et al.*[18] using an absorption coefficient of 32.4 mM^{-1} cm^{-1}, for the difference 555 nm max. minus 575 nm min. of dithionite reduced form against oxidized as reference.

All the spectrophotometric measurements were carried out at room temperature using a Beckman Acta V spectrophotometer, only the reactions with various ligands before the PMR measurements were followed using a Perkin-Elmer 124 spectrophotometer.

The longitudinal proton magnetic relaxation time, T_1, was measured at 24 MHz by 180^0-t-90^0 pulse sequence as described in ref. 7.

Owing to the large free space above the small (~0.15 ml) sample and due to the temperature gradients in experiments above room temperature, some water-vapour condensation was observed in samples in ordinary NMR-tubes, resulting possibly in a change of the sample concentration. For precise temperature studies special PMR-tubes were made with a ground-glass stopper above the sample space of ~0.2 ml. This device could be kept in contact with the outer atmosphere (CO or air), or closed by turning the ground stopper whose inside served as thermocouple well.

Sample Characterization

Usual difference spectra were used in order to characterize samples at different stages of preparation. One such example is shown in Figure 1 for the microsomal

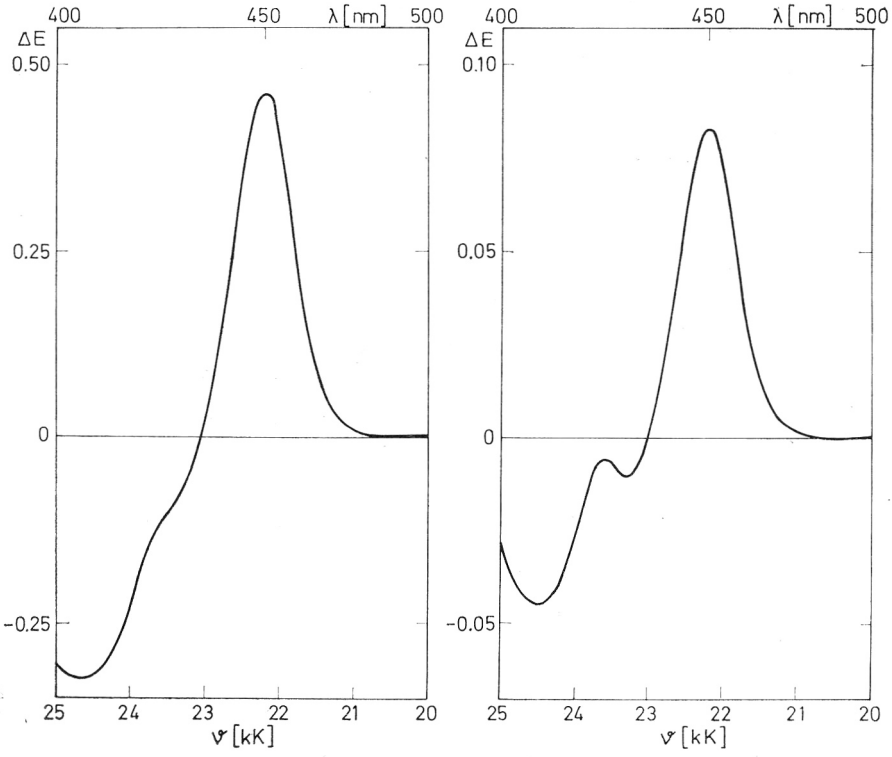

Figure 1. CO-difference spectrum of the microsomal cytochrome P-450 (left) and of the solubilized form (right) after concentration by ultrafiltration.

and solubilized sample. ESR-spectra were also used, Figure 2 showing a typical spectrum obtained with microsomal preparations. The same characteristic ESR--spectra were obtained with frozen solubilized samples, but in the more concentrated solubilized cyt P-450 the line at g = 2 was a little stronger. Therefore it was explored if this may be due to admixtures of copper, manganese, or non-haem iron. A

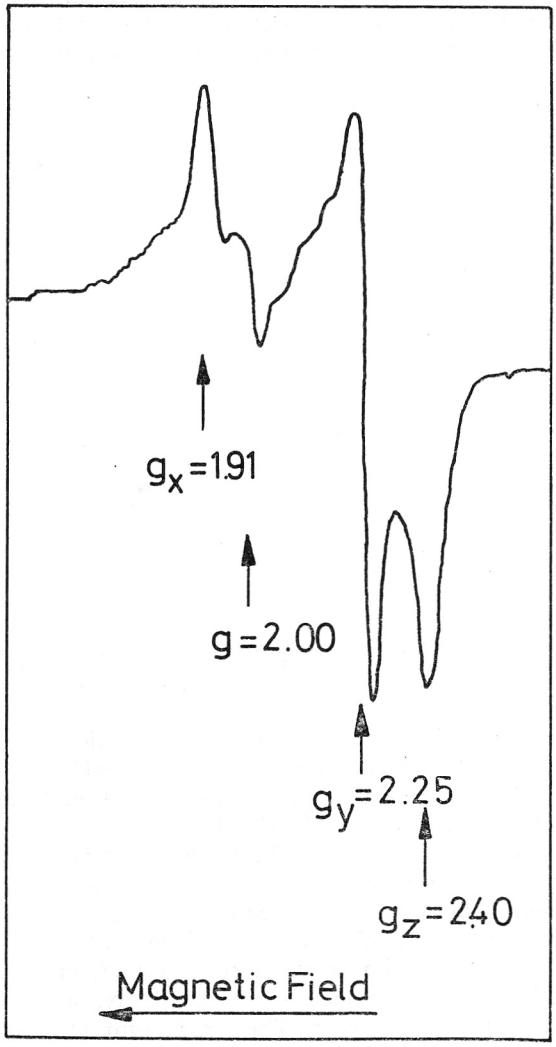

Figure 2. ESR spectrum of cytochrome P-450 from microsomes; temperature 77 K, modul. ampl. 80 Gauss.

qualitative analysis by emission spectroscopy of the reconstituted (cyt P-450+P-450 reductase) and microsomal sample showed only the presence of Cu and Mn in amounts certainly less than 1/100, and possibly 1/1000, of the iron content.

The determination of total iron content in microsomes and in the solubilized sample by atomic absorption spectroscopy showed that in both about 70% (±3%) of *total iron* was accounted for by total *haem*-iron.

RESULTS

Figure 3 represents a PMR titration of a solubilized P-450 sample with KCN. Three aliquots of the P-450 solution were used for stepwise addition of different KCN stock solutions, so that in each run the final sample dilution was not more than 30%. Each particular change in concentration due to KCN--additions was accounted for when calculating the total relaxation rate (ordinate in s^{-1}). The observed diminuation of rates is most probably due to a

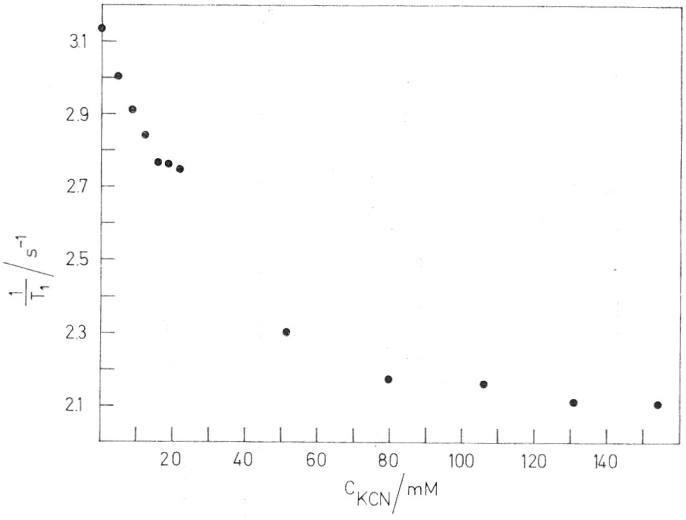

Figure 3. The longitudinal proton magnetic relaxation rates, $1/T_1$, (ordinate) of the solubilized cyt P-450 (0.625 mM in P-450 haem, 0.180 mM in P-420 haem, and 0.086 mM in cyt b_5 haem, initial conc.) in dependence on concentration of KCN added (abscissa), at 24.5 ± 0.5 °C.

decreased accessibility of the haem-iron when the cyanide ion was bound on it. The dissociation constant was estimated to be ~ 30 mM KCN.

Data shown in Figure 4 result from isotopic dilution of microsomes (open circles) and a solubilized P-450 sample (dots). The dialysis tubing was fixed at the end of a glass rod. Approximately 0.2 ml of the given sample was put in, and the free end of the dialysis tubing was tightened without leaving air inside. The microsomal sample was immersed in about 8 ml of D_2O containing the same salt concentration as the microsomal solvent (H_2O). After the start of dialysis the PMR rate of the sample withdrawn from dialysis and inserted into the NMR-probe was measured successively, at room temperature; dialysis itself was performed at cold. The final point with only 20% protons left from the original sample (100% in protons) was obtained after 46 hours of dialysis. The proton amount in the sample was determined comparing the corresponding amplitude of proton magnetization at the largest pulse-delay of the sample and a standard of ordinary water, so the increase in the amplification necessary in these measurements was taken into account. The dots were obtained in a similar way, only the dialysis was performed against 2.5 ml of D_2O containing also 20% glycerol distilled earlier from D_2O. The dialysis was much faster in case of this solubilized P-450 sample, having been accomplished within 24 hours, the largest difference having taken place within the first

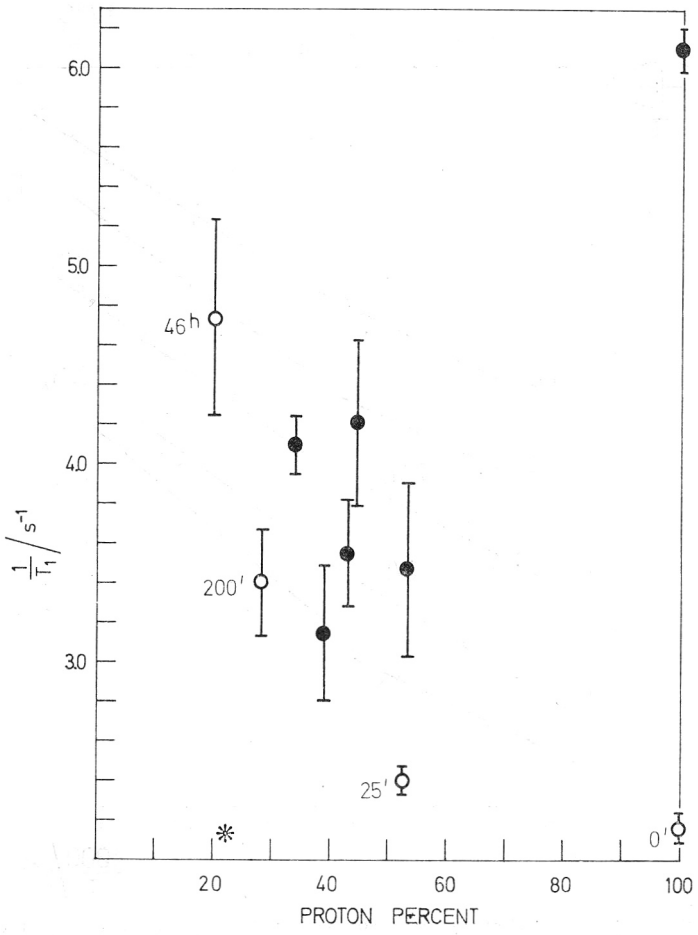

Figure 4. The PMR rates (ordinate) of microsomal (circles, with the times indicating the duration of dialysis against D_2O) and solubilized (points) P-450 in dependence on the H_2O/D_2O composition of the solvents (abscissa), at room temperature. The PMR-value denoted by the asterisk is due to the originally deuterated microsomes (see Expl.) to which 19% H_2O was added and PMR-rates measured three times at intervals within 195 minutes.

15 min, while a similar drop in proton content for the microsomes required 25 min.

The Arrhenius plots of the temperature dependence of the PMR rates as obtained from the direct measurements with original (marked a) and dithionite--CO-reduced samples (marked b) are given in Figure 5. The straight lines are least-squares best fits with significantly different slopes for the low and high temperature regions. The data 1/a are derived from the solubilized sample and were obtained in a warming and recooling cycle without measurable systematic deviations that would indicate any hysteresis. All the »b«-data were obtained with samples thoroughly flushed by a CO-stream before and after addition a few grains of dithionite. The colour change was always obvious, and the microsomes appeared less opaque.

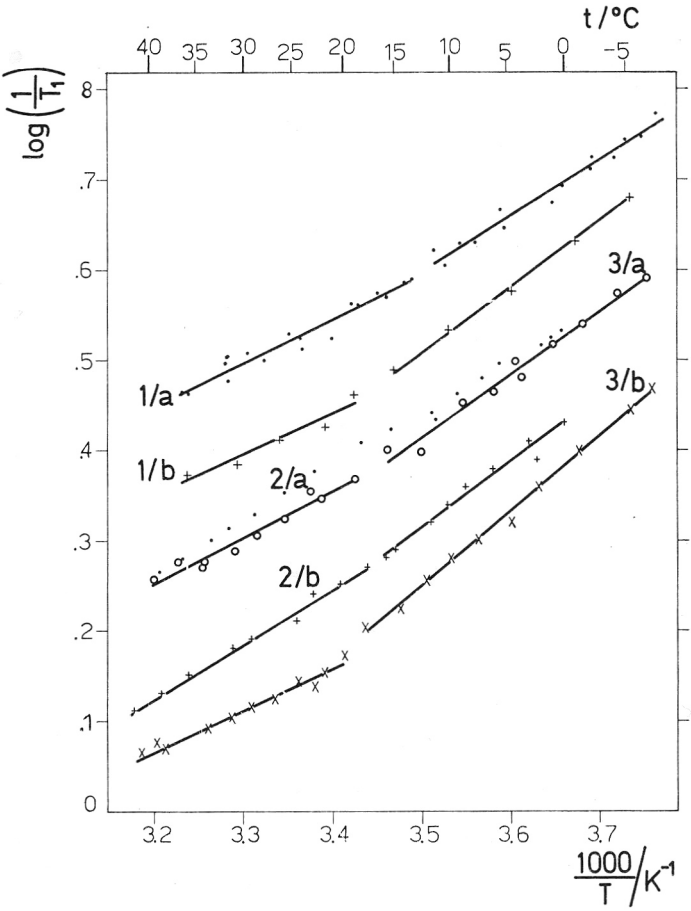

Figure 5. Arrhenius plots for PMR rates of solubilized (1), microsomal (2) and reconstituted (3) P-450 samples. (See text for the corresponding concentrations.) The data for the original samples are designated *(a)*, those for the CO-dithionite reduced *(b)*.

The *molar* relaxation rates plotted in Figure 6 were computed from Figure 5. In doing so we first subtracted the PMR rates (given in reciprocal seconds) of the measurements with the reduced samples *(b)* from the corresponding original ones *(a)*. The PMR contribution of admixed cyt b_5 was assumed negligible owing to its very closed haem-pocket with the iron coordinated to the two histidyls as well[19]. On the other hand, our PMR control measurements with the original solubilized sample converted from some 75% P-450 to 75% P-420 by sodium deoxycholate (NaDOC) did not indicate any change in PMR rates. Therefore, the a-b incremental relaxation rates from Figure 5 were normalized in each particular case by dividing them with the corresponding P-450 + P-420 haem-content. *i. e.* by 0.805 mM for the solubilized sample (1), by 0.324 mM for the microsomes (2) and by 0.403 mM for the reconstituted (solubilized + reductase) sample (3). The molar relaxation rates in Figure 6 for solubilized (1) and reconstituted (3) samples were obtained from differences

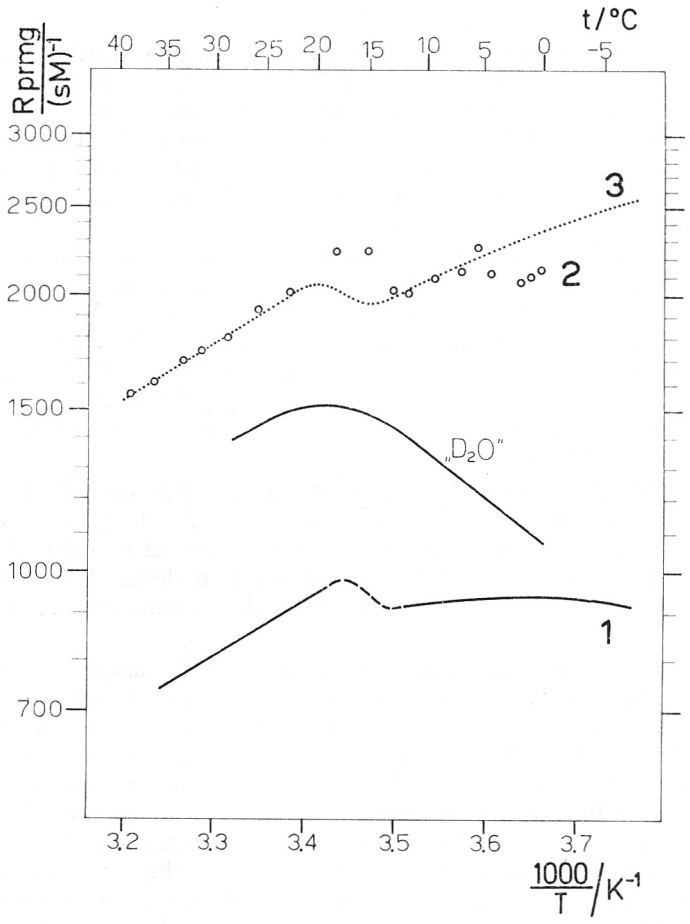

Figure 6. The molar paramagnetically induced relaxation rates derived from Figure 5. with the same numbering of the samples. »D_2O« indicates similar temperature dependence for the microsomal P-450 prepared in D_2O, to which 19% H_2O had been added (the corresponding original and CO-dithionite reduced data are not shown in Figure 5.).

of the corresponding a and b straight lines. The points given for the microsomes (2) in the same Figure were deliberately obtained by subtracting from each particular experimental point 2/a the corresponding value read off the straight lines (2/b in Figure 5).

The molar relaxation rates marked by »D_2O« in Figure 6 were obtained from measurements with a completely deuterated sample of microsomes to which 19% ordinary water has been added. The original, D_2O-sample, was prepared in the usual way (see Experimental) with replacement of H_2O by D_2O at al stages of preparation. The control *proton* magnetic relaxation measurement for that sample showed only 3% residual protons (taken into account in determination of H_2O in partially deuterated microsomal samples) contained probably within the membraneous matrix.

DISCUSSION

There are two points to be considered before a quantitative evaluation of the PMR results in structural terms could be attempted. Firstly, what is the origin of the 30% excess iron in both the microsomal and in the solubilized samples, *i. e.* how it may contribute to the measured paramagnetic relaxation rate? Secondly, if these rates are due to the *low-spin* P-450 haem-iron, they appear to be too large by what is known of such iron in more »conventional« haemoproteins.

The possibility that the excess iron could be haem-iron of haemoproteins other than those common to the P-450 system can be discarded because the sum of the components equalled the concentration determined as whole haem--iron. Besides, the KCN PMR-titration (Figure 3) of the solubilized sample resulted in a dissociation constant (see Results) of 30 mM KCN. This is larger than the corresponding constant for myoglobin[20] by three orders of magnitude, but it is in good agreement with that obtained by difference spectrophotometry for various cyt P-450's[25].

Additionally, if it were haemoglobin (or myoglobin) the paramagnetic PMR-rate could not be measured at all (see later). The large difference in the dissociation constant for the two kinds of haemoglobins is rationalized by the notion that in haemoglobin the sixth-ligand water molecule has to be replaced by a cyanide ion, while in P-450 there is the nonmercaptide (imidazole?) ligand[21].

The excess-iron amount is about the same for both samples, *i.e.* for the microsome and the solubilized one. The preparative procedure for the former involved a very thorough washing procedure starting with an addition of 2 mM EDTA. The difference in composition of the final solvents (glycerol added for the solubilized sample) should have caused a different surplus iron content if some of the chemicals were Fe-contaminated. The solubilized sample was also »titrated« with EDTA up to the concentration exceeding 2.5 times that of the non-haem iron, but no decrease in the relaxation rate was observed. The results in Figure 6 show twofold larger relaxation rates for the microsomes compared to the solubilized sample; this cannot be explained by their equal amount of excess iron.

It remains to consider that the surplus iron-ions are bound to the protein(s). If *(a)* they all are »chelated« by suitable amino acid residues (or somehow burried inside the protein) their paramagnetic contribution to the total relaxation rates would be negligible owing to a faint »outer-sphere« dipole--dipole interaction with solvent protons. If, on the other hand, the first coordination spheres of these ions are *(b)* partly accessible to solvent molecules, one would expect them to have a rather high affinity for the cyanide ion, so that the titration curve in Figure 3 ought to have shown at least one additional step, which is not observed.

A special case would be the contamination with ferritin[22]. Its molecule, when filled completely with the iron-core within the protein shell, has a molecular weight of 860 000. With this molecular weight (2800 to 4500 iron atoms are contained within such a unit) the 30% surplus iron in our samples would require much too small a fraction ascribed to ferritin to be detectable in analytical ultracentrifugation. The iron core of ferritin is a closely packed crystal structure of iron and oxygen atoms without any, or with a very slight

content of water molecules. This contradicts the free accessibility of the core for solvent protons. The exchange of iron between ferritin and solvent is a slow process, so that the presence of free iron from this source is not likely (see also above concerning the CN^- binding.) The »average« spin for the ferric iron in ferritin is about $S=3/2$, suggesting in fact a spin-state mixture of high and low spin iron-ions. Nothing is known about their electron relaxation times, but the protein shell must be born in mind, if a usual magnetic dipole-dipole interaction between the water protons and the iron of the ferritin core is assumed. Its width is about 22 Å. Hence a drastic reduction of the paramagnetic induced PMR-rates would be expected owing to their r^{-6} dependence on the proton-to-iron spin distances.

We cannot yet specify the exact nature of this excess iron content, but it is apparently ineffective regarding the measured paramagnetic rates. In other words, we believe that the measured paramagnetic relaxation rates are indeed due only to the P-450 haem-iron. Other evidence supports this conclusion: For instance, if these rates were due to the unidentified excess iron, it is difficult to explain why the rates for the microsomes and the solubilized sample differ by a factor of two, the excess-iron content being the same in both. Besides, the paramagnetic relaxation rates of our solubilized sample are very similar to those measured with the soluble P-450 from *Pseudomonas putida* known to contain only P-450 low-spin haem-iron[8].

It has been shown by Estabrook and his coworkers in a series of papers (see ref. 23) on liver microsomes that a slight amount of NADPH will enhance the P-450 reduction in the presence of NADH, while the reverse is not true. The conclusion was that while both of these reducing agents are present *in vivo*, their reducing action upon the ferric haem-iron of P-450 is coupled: NADPH appears to be very specific for the action of the flavoprotein reductase dependent on it. We therefore performed reduction of P-450 ferric iron by either NADPH or NADH, under a CO-atmosphere at room temperature. The paramagnetic contribution to the relaxation rates eliminated by NADPH-reduction was identical to that obtained usually with dithionite, a certainly less specific reducing agent. The result for the NADH reduction was a lower paramagnetic relaxation rate in accord with the expected less efficient reduction. The good agreement between PMR rates obtained from dithionite and NADPH measurements is indeed reassuring from the purely experimental point of view. However, electrochemical reduction of inorganic Fe^{3+} into Fe^{2+} has been accomplished (by Dr. F. Scheller from the Division of Biokatalysis, C. I. M., Ad.W., G. D. R.) in the presence of NADPH and NADPH-dependent reductase. Thus, the cyt P-450 PMR-values obtained from the NADPH measurements cannot be taken to prove that non-haem iron does not contribute to the measured relaxation rates.

The final clear-cut answer could be obtained with similar measurements on samples free of any excess iron[24], but, it seems presently permissible to start with the working hypothesis that the measured paramagnetic relaxation rates in this paper are indeed due to the effects caused by the haem-iron of cytochrome P-450.

The next point to consider is that of the *molar* paramagnetic relaxation rates in Figure 6, which, for preponderately low spin haem-iron in our samples appear to be much too high (see later).

The PMR-rates with soluble bacterial low-spin P-450 were also quite high[8] and comparable with ours, so that it is imperative indeed to analyze different explanations of such an observation.

The *molar* rates were obtained by dividing the purely paramagnetic contribution with the haem concentration (see Results). The latter was obtained from analytical determinations assuming a physically homogeneous sample. The solubilized samples may indeed be homogeneous, although the formation of micelles cannot be excluded owing to the presence of hydrophobic portions in these 660000 daltons »macromolecules«. Microsomes, on the other hand, are certainly not homogeneous; the solvent is encaged within them and possibly behaves differently from that on the outside. The problem is, therefore, to determine the extent to which the protons, whose relaxation is measured in these experiments, are equally exposed to the influence of the paramagnetic centres.

Figure 4 shows PMR-measurements during the dialysis of microsomes and solubilized P-450 against D_2O. The PMR-rates depend on the total (per cent of initial) content of protons whose relaxation can be measured, *i. e.* from the solvent phase. The points for *microsomes* indicate more than a twofold *increase* in the total relaxation rate after the equilibrium had been achieved, with about 20% of initial protons nonexchanged by dialysis. This implies that a considerable fraction of the intramolecular water is nonexchangeable which was confirmed by the reverse experiment, *i. e.* starting from the fully deuterated microsomes: measurements with this sample after addition of 19% H_2O yielded PMR-rate as indicated by the asterisk in Figure 4. It remained constant within 195 minutes. The difference between that value ($D_2O \rightarrow + H_2O$) and that from the dialysis experiment ($H_2O \rightarrow + D_2O$) is a strong evidence that there is a highly structured immobilized water within the microsomes, independent of the outer bulk solvent. Results of independent studies on permeability of microsomes[42] are consistent with this conclusion.

Therefore, if all the paramagnetic centres were located inside the microsomes, the *paramagnetic* molar relaxation rates should be calculated on the basis of a five time larger concentration within the microsomes. The question therefore arises about the location of the paramagnetic centres. In Figure 6 we present results obtained with fully deuterated microsomes to which 19% H_2O was added. The molar paramagnetic relaxation rates shown there were obtained in the usual manner, *i. e.* by subtracting first the rates measured with the same deuterated sample (+19% H_2O) in the reduced, CO-state. The values around room temperature for the deuterated sample are about 75% of those for the H_2O-microsomes. Hence, most, if not all of the paramagnetically induced relaxation rate in the normal microsomes must be due to the magnetic dipole interaction of proton spins with *paramagnetic centres located on the outside surface of the microsomes.* The latter are therefore an integral part of the bulk solvent (80%) as far as the PMR-measurements are concerned. As the concentration correction required ($\times$ 0.8) is not serious we preferred to reproduce data using the actual concentrations determined for the total sample volume. (For lack of material we were not able thus far to determine directly the *paramagnetic* contribution to the »inside relaxation rates« of those nonexchangeable 17% protons.)

In the course of D_2O-dialysis the *solubilized* sample showed a *decrease* in the PMR-rates (see Figure 4). This can be ascribed to the effect of isotopic

dilution, because the dipole-dipole induced relaxation within each water molecule is diminished. For lack of fully deuterated glycerol we did not achieve full exchange of protons for deuterons. Nevertheless the data in Fig. 4 indicate that the solubilized sample is indeed homogeneous in the sense that there is only one measurable (physical) phase of relaxing protons under our experimental conditions, *i. e.* the bulk solvent. Hence, the molar paramagnetic relaxation rates, calculated on the basis of the total concentrations, are correct the system behaving as a true (macro)molecular solution.

The preceding conclusion about two different phases of the solvent in samples of microsomes, without fast proton-communication between these phases implies that the free induction decay, or the magnetization curves measured by the 180°—90° pulses, are composed of two independent decays characterized by the corresponding two T_1-relaxation times. The experimental verification of this would depend on the difference between the two relaxation times as well as on the fraction ratio of the two phases. Using estimates of limiting T_1-values, 0.67 s for the bulk phase (80%) and 0.20 s for the microsomal phase (20%), the corresponding magnetization curve was constructed. The deviation from an approximate straight-line plot on the semilog scale for one »common« T_1 was within our experimental precision in view of the diode-detection system which introduces errors in the zero-magnetization range. It is beyond doubt that the overall T_1's calculated from our actual measurements cannot have a systematic error larger than 10%, so that the data given in Figures 5 and 6 are valid for the present discussion.

We are therefore confronted with the problem of high relaxation rates induced by *low-spin* haem-iron of cytochrome P-450 observed in the present study as well as in that on the soluble bacterial P-450[4]. With τ_c, the correlation time ($\equiv$ electron-spin relaxation time τ_s) in Solomon's theory[26] for magnetic dipole-dipole interaction, equal to 10^{-12} s (see ref. 27, p. 82) for low-spin haem-iron and with the corresponding spin-value (1/2 v. 5/2 for high-spin) the smallest possible iron-to-proton distance (yielding therefore the largest rates) would result in PMR-rates *two orders* of magnitude smaller than those observed. If we accept the magnetic dipole interaction as the only mechanism determining the PMR-times in our system(s) the obvious conclusion is that the correlation time, τ_C ($= \tau_S$), is much longer than 10^{-12} s. Correlation times of about 10^{-10} s and longer have been measured for methaemoglobin derivatives, aside by PMR[28] also by ESR-line-width at room temperature[29], but for the *high-spin* iron. No room-temperature ESR-spectra of the low-spin cytochrome P-450, or of more »ordinary« haemoproteins, have yet been published. The conclusion about the preponderately low-spin character of P-450 is based upon ESR-measurements with frozen samples at 77 K or even at 1.2 K[21]. Griffin and Peterson[8] obtained a $\tau_C \cong 4 \times 10^{-10}$ s for both the high- and low-spin states of their bacterial P-450, by an extrapolation procedure of the ESR-line-widths measured on frozen samples at rather low temperatures. In view of the (puzzling) fact that the visible spectra are practically *independent* on temperature[30] and that the magnetic susceptibility of the frozen bacterial P-450 obeys the Curie law[31] it may well be that the ESR-extrapolated estimates are true correlation times for the relaxation mechanism at room temperature. The relaxation rates obtained in the present study with the microsomal and reconstituted samples ($\sim$ 2000 s^{-1} M^{-1}) are one half of the highest rate for an aquomethaemo-

protein ever measured — that for acid cytochrome c[32]. In the latter, two water molecules are directly coordinated to the haem-iron, while in cyt P-450, at best, only one water molecule could be expected in the first coordination sphere of iron. Hence, in order to rationalize the observed PMR-rates with microsomes one must assume that the haem-iron is completely in the high-spin state with the same, extremely efficient relaxation mechanism as for cytochrome c. This is definitely contrary to all our findings with the microsomal preparations in this study, *i. e.* that the haem-iron was preponderately low-spin. The final conclusion in this respect could only be reached by measuring the PMR dispersion in an as wide as possible range of magnetic fields. Should it turn out that the correlation time is of the order of 10^{-10} s, it would confirm the applicability of the magnetic dipole relaxation theory to this complicated system enabling thus quantitative conclusions to be made and hence introducing the natural, but *low-spin* ferric iron of cyt P-450 as a stereochemical probe of substantial biological interest.

With the present state of the art it does not seem warranted to discuss the PMR-data in quantitative, strucural, terms.* Regarding relative comparisons of the PMR rates from solubilized P-450 measured with different additives to the system, our experiments are not yet conclusive, though they seem to indicate that *(a)* either the PMR-rates are not sensitive enough, or *(b)* that there is no such conformational change which would alter the rates when the predominately (70%) P-450 sample is converted to more than 70% P-420 on addition of sodium deoxycholate (NaDOC). The same rates were obtained also when to both of these forms (P-450 and P-420) KCN was added, but at a concentration about three times smaller than the dissociation constant indicated by the titration (see Figure 3). One of type-I substrates, methphenethamine hydrochloride (Spasman®), similar chemically to benzphetamine, did not produce significant changes in the PMR rates, either, but a substrate type-II, aniline, seems to lower the relaxation rates slightly.

Preliminary measurements by our »stereochemical PMR-titration« method[7] using deuterated solution in which only the aliphatic protons of the glycerol

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* Such an attempt in ref. 8 is liable to serious criticism for the following reasons: Although the camphor-bound P-450 results (ref. 8, Figure 6) may be analyzed in terms of the existing theory because the haem-iron is *high-spin,* the outer-sphere mechanism assumed by the authors is not proven. A direct experimental distinction can be made between the outer-sphere and the fast-exchange mechanism by the »PMR-stereochemical titration«[7]. If such measurements showed that fast-exchange mechanism were operative, the interspin distance(s) could justifiably be calculated. On the other hand, if the outer-sphere mechanism were indeed true, in this case no structural parameters like interspin distances can be calculated for simple reason that the equation used[8] was obtained by integration assuming spherical symmetry around the paramagnetic centre, which certainly is not the case with haemoproteins (see ref. 7). The original, unreacted P-450-data of the same study[8], with which our values are in agreement, most probably could be ascribed to the so-called fast-exchange relaxation mechanism, but they pertain to the *low-spin* haem-iron. The authors calculated 2.1 Å for the proton-to-iron distance for the fast-exchanging protons in the first coordination sphere of iron. From the X ray data on the location of water-oxygen in aquometmyoglobin[33] this distance could not be shorter than 2.6 Å. Their conclusion that one of the axial ligands of the haem-iron must therefore be a water molecule like in aquometmyoglobin or the same derivative of haemoglobin is not yet confirmed and is difficult to reconcile with our PMR-determination of the CN^- dissociation constant to P-450 which is so extremely large compared to myoglobin.

served as the »PMR-probes« suggest strongly that the type of the PMR mechanism is not altered, remaining in all likelihood that of fast-exchange, as concluded by Griffin and Peterson[8] for the bacterial low-spin P-450. The absolute values of the PMR-rates from the aliphatic glycerol protons were about 65% of those for the original (solubilized) sample in which the rates are due to the water-protons. This is consistent with the expectation that larger glycerol molecules cannot approach the haem-iron as close as water molecules do. From our experience with other, but high-spin, haemoproteins[34,35] the observation of a fast-exchange mechanism of a glycerol molecule is compatible with a rather accessible haem-iron, *i. e.* a very open »haem-pocket«, if there is a haem-pocket *at all* of the type found in myoglobin. On the contrary, the ESR-spectra obtained with the spin-label firmly bound to the haem-iron of rat-liver P-450 *microsomes* led the authors[5] to conclude that the haem is situated within a very tight hydrophobic pocket. The distance from the iron-ion and the free radical *end* of this spin-label (from which the conclusions were derived) is about 10 Å, and it may just as well be located within a part of the protein matrix restricting its accessibility. While valuable conclusions were reached about other aspects of the haem-environment in this ESR-study[5], it seems to us that the free-radical accessibility of such a spin-label covalently bound to haem-iron should be related with caution to the actual immediate haem-environment which, in our approach with PMR is in its native state, *i. e.* unobstructed by any artificial ligands to the iron-ion.

The three constituents accomplishing the hydroxylation or demethylation of a variety of substrates, cytochrome P-450, NADPH-dependent P-450 reductase and a phospholipid are membrane bound, the first two being membrane-bound enzymes. It is therefore important to learn something about the structural changes, if any, in the vicinity of the haem-iron of the main constituent, the mixed function oxygenase cytochrome P-450, at different levels of association with other constituents. The PMR-data in Figures 5 and 6 are instructive in this respect notwithstanding the main unsolved problem, that of the actual relaxation mechanism of protons interacting with the *low-spin* iron of cytochrome P-450. As we have already pointed out in the preceding discussion, these PMR-data are expected to be quite valid for relative comparisons, reflecting an overall conformational situation around the haem-iron in P-450. The PMR temperature dependence in Figures 5 and 6 has been measured with great care (see Experimental), so that the following main conclusions may be drawn:

1. There are two temperature regions characterized by two distinct Arrhenius plots (see Figure 5) for the original microsomes, the solubilized sample and for the partially reconstituted one and for each of them in the reduced--CO-state, as well. The kink-temperature in these plots is between 13 and 20 °C. Whatever the real cause of this kink (it may be, quite probably due to the well-known membrane phase-transitions), it demonstrates the sensitivity of the solvent-proton magnetic relaxation to slight structural alterations within the macromolecules under study.

It is of more specific interest to see if such structural alterations are also sensed *via* the *paramagnetic* part of the relaxation rates, *i. e.* after subtracting the diamagnetic (CO-reduced) contribution. It is clear from Figure 6 that this is the case for all three samples. Again, with reference to our experience with (high-spin) myoglobin and haemoglobin bound to solid matrices like latex[36]

and Sephadex resins[37] it is not surprising that the paramagnetic PMR rates are sensitive to structural alterations in case of cyt P-450. However, in none of the other haemoprotein cases studied have we observed such kinks in Arrhenius plots as of the kind found in the present work. It thus seems possible to study by PMR, at least in a qualitative way, changes in this kind of »phase--transition« when the system interacts with substrates, or other (small) molecules.

2. The cause of the described PMR-discontinuity most probably lies in the phospholipid part, certainly of the original microsomes, but also in the solubilized and in the reconstituted samples, which were not completely free of lipid. The content of phospholipids accompanying the proteins in the solubilized samples is much smaller than in the intact microsomes. In spite of this, the solubilized P-450 shows the paramagnetic PMR temperature-discontinuity (see Figure 5/1a). Further PMR study will have to show whether the temperature--discontinuity sensed by the paramagnetic relaxation rates is induced by the phospholipid phase-transition common to membraneous structures, but it is the likely case. If so, it means that the first-neighbour protein/phospholipid interaction is most responsible for the protein conformation (changes) around the haem-iron. Moreover, as evidenced by the very close similarity of the paramagnetic PMR data for the original microsomes and the partially reconstituted sample (Figure 6, 2 v. 3), it appears that the cyt P-450-protein and the reductase-protein are in very close interaction. By this interaction the paramagnetic PMR rates of the solubilized P-450 are changed to the higher ones of the original microsomes (see Figure 6, from 1 to 3). In other words, the missing part of the phospholipid may not have such a crucial role in defining the spatial configuration around the haem-iron in cyt P-450 judged by the sensitivity of the PMR method. Further experiments along these lines are in progress in our laboratories, but other experimental and theoretical approaches appear to be consistent with the picture derived from the present PMR data. For instance, Stier and Sackman[38] used spin-labels as substrates for cyt P-450--microsomes combining in such a way the information on the function of this membrane-bound enzyme system with those on the membrane structure. For the lipid-soluble spin-label a break in the Arrhenius plot was found at 32 °C, while the lipid phase was described as non-homogeneous, with about 20% of it rather rigid, surrounding the protein molecules. Ruf and Duppel[39] came to the same conclusion using other spin-labels with hepatic microsomes. They observed a break related to the rigid-phase lipid corresponding to the Arrhenius kink at 20 °C for the *o*-dealkylation by microsomes[40]. In a theoretical approach using the molecular field calculation Marčelja[41] concluded that the interaction between protein molecules in membranes does not extend appreciably over more than two intervening lipid molecules. It therefore seems that the PMR observations in this report reflect conformational changes at the »active site« of a membrane-bound enzyme. These changes may be induced from the »rigid« part of the lipid phase in the membrane, without any external structural perturbations.

Acknowledgement. The work has been carried out with the technical assistance of H. Grill, D. Bergmann and V. Bračika. The preparation of the reductase as well as the activity determination with the reconstituted samples were done by Miss D. Baess. Thanks are due to Miss M. Kajzer (Institute »Ruđer Bošković«, Zagreb) and to Dr. Schmidt (Central Institute for Optical Spectroscopy, Berlin) for carrying out

the spectroscopic analyses. S. M. is much obliged to Dr. R. Estabrook for making available the manucript of ref. 8. prior to publication. The authors are grateful to the referees for valuable comments. This research was made possible through the financial support from the PL-480 Program (Project 02-004-1 between NIH and IIZ) and the Interchange Program of the Academy of Sciences of GDR and the Council of Academies of SFRY.

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SAŽETAK

Jetreni citokrom P-450. Istraživanje mikrosomalnog, solubiliziranog i djelomično rekonstituiranog enzimskog sistema protonskom magnetskom relaksacijom

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Neposredna okolina željeza hema u citokromu P-450 karakterizirana je mjerenjem temperaturne zavisnosti brzine longitudinalne magnetske relaksacije protona iz otapala (vode). Doprinos toj brzini relaksacije (BR) od paramagnetskog željeznog iona određen je iz razlike BR za originalne uzorke i one reducirane (uz CO) ditionitom ili pomoću NADPH. Iz opadanja BR pri titriranju kalijevim cijanidom dobije se konstanta disocijacije od oko 30 mM KCN, što je za tri reda veličine veće od te konstante za metmioglobin, odnosno slobodni feri ion. Zaključeno je da su izmjerene BR najvjerojatnije posljedica prisustva željeza hema od P-450 (i P-420), dok 30%-ni suvišak željeza nađen i u mikrosomalnom i solubiliziranom P-450 ne pridonosi mjerenoj brzini relaksacije. Izotopskom (D_2O/H_2O) se dilucijom BR povećavaju za mikrosome, a smanjuju za solubilizirani P-450. Mikrosomi zadržavaju 17% uklopljene H_2O, a najveći dio njihove paramagnetske BR potječe od paramagnetskog željeza smještenog na mikrosomima izvana. Ovo je potvrđeno mjerenjima na deuteriranom uzorku, kojem je bilo dodano 19% H_2O. Prema tome je solubilizirani P-450 homogen (otopina) s obzirom na BR, dok mikrosomi nisu.

Paramagnetske BR su začudno visoke za pretežno nisko-spinsko željezo u ovim uzorcima i za sada su moguće samo usporedbe pristupačnosti željeza hema, bez kvantitativne analize podataka. Tako npr. usporedba BR dobivenih mjerenjem preko protona vode i onih od alifatskih protona deuteriranog glicerola (u deuteriranim uzorcima P-450) ukazuje na vrlo pristupačno željezo hema. Arrheniusovi grafovi pokazuju jasno dva temperaturna područja s lomom između 13 i 20 °C kako za originalne, tako i za sva tri reducirana uzorka. Isto vrijedi i za paramagnetske BR, koje su dva puta veće za mikrosome od onih za solubilizirani P-450. Kad se ovaj posljednji rekombinira sa solubiliziranom (NADPH)reduktazom, BR su praktički iste s onima od originalnih mikrosoma. Čini se, dakle, da odsustvo dijela fosfolipida u rekonstituiranom uzorku nije bitno za konformaciju oko željeza hema. Vjerojatnije je, da se funkcionalna (?) konformacija ostvaruje već interakcijom prvih susjeda (protein P-450+protein reduktaze) razdvojenih možda samo jednom molekulom fosfolipida. Ta je konformacija osjetljiva na strukturne promjene membrane kao cjeline, oko temperature »prijelaza«.

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i
IMUNOLOŠKI ZAVOD ZAGREB

Prispjelo 25. rujna 1975.