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Conference Paper

## Mixed Solvents as Tools to Study the Structural Aspects of Cytochrome P450 in Solution at Sub-Zero Temperatures\*

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Cytochromes P450 from various sources are known to have mixtures of different heme-iron electronic configurations, partly in thermodynamic equilibrium, reflecting different configurations of the protein.

Organic solvents (such as ethylene glycol or glycerol) as well as temperature changes have a marked but reversible effect on the thermodynamic parameters of these equilibria in the *fluid state*.

The solvent and temperature perturbation of the conformational or spin state equilibrium have been investigated on various redox states of cytochrome P450 in order to gain some insight into the structure of the heme pocket.

### INTRODUCTION

Variations of optical spectra have been largely used by many authors as natural probes of either slight changes in the near heme environment or larger changes of the whole protein<sup>1-3</sup>. The importance of performing such studies in the liquid state is especially obvious when spin state changes are involved, since most measurements are usually performed at very low temperatures where the strains and distortions imposed by the frozen medium could introduce many artefacts<sup>4</sup>. Furthermore, it is usually difficult to establish a clear correlation between the structural data obtained at liquid nitrogen temperature and the enzymatic reactions performed at room temperature.

The evolution of the spectral and spin state changes in various redox states of cytochrome P450 have been studied in a wide range of subzero temperatures and in fluid hydro-organic media, that is under conditions where their reactions have been simultaneously recorded<sup>5,6</sup>.

### EXPERIMENTAL

All experiments were performed in 1:1 (v/v) mixtures of aqueous buffer and ethylene glycol. The protonic activity of such mixtures has been determined for each buffer as a function of temperature down to the freezing point<sup>7</sup>.

Spectra are recorded on an Aminco-Chance DW 2 or a Beckman spectrophotometer adapted in such a way that the two cells may be thermostated at any chosen temperature between +40 °C and -100 °C with a temperature difference of less than 0.5 °C, as already described<sup>8</sup>.

\* Presented by P. Debey at the Scientific Conference »Cytochrome P450 — Structural Aspects« (held in Primošten — Yugoslavia, 6—10. October, 1976).

## RESULTS AND DISCUSSION

*Ferric Cytochrome*

The spin state of the ferric microsomal cytochrome P450 is known to be markedly dependent on the chemical used for its induction, as well as on the presence and nature of bound substrates<sup>9,10</sup>. A useful model for the investigations of microsomal P450 is the bacterial cytochrome. Nearly purely low spin (LS) when in the free ferric enzyme form ( $m^0$ ), it becomes almost totally high spin ( $m^{0s}$ ) on binding of the substrate ( $m^{0s}$ ) as evidenced by the optical 417 nm  $\rightarrow$  392 nm shift recorded at room temperature<sup>11</sup>. However, the HS content was only 60% when measured at 12.5 K<sup>11</sup>. The thermal equilibrium between spin states, initially proposed to explain this discrepancy<sup>12</sup> has been questioned recently<sup>13</sup>. Furthermore, the HS content as measured by EPR has been shown to depend, in a way, on the preparation<sup>13</sup>.

The existence and evolution of the temperature dependent spin states equilibrium of  $m^{0s}$  can be easily measured by optical spectroscopy in fluid medium, as shown by the temperature difference spectra in Figure 1. In these

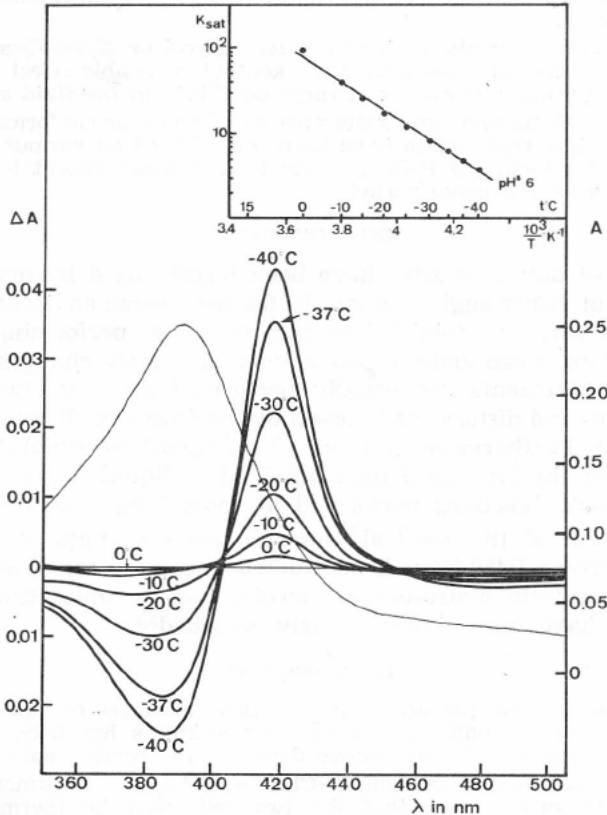


Figure 1. Temperature induced spin state change in the camphor saturated bacterial cytochrome P450 ( $m^{0s}$ ). Solvent: 1:1 v/v mixture of 0.2 M cacodylate buffer pH 5.5 and ethylene glycol containing 0.1 M KCl and 3 mM camphor as final concentrations. Absolute spectrum taken at +20°C. Aminco-Chance DW2 spectrophotometer. For difference spectra, the reference is maintained at +15°C while the sample is thermostated at various temperatures indicated. The insert shows the van't Hoff plot of the calculated HS/LS ratio.

experiments the solvent is a 1 : 1 (v/v) mixture of 0.2 M cacodylate buffer, pH 5.5, and ethylene glycol, the pH\* of which is 0.5 units above that of the purely aqueous buffer at room temperature and varies only slightly with temperature<sup>7</sup>. The insert in Figure 1 shows that the Van't Hoff plot of the equilibrium constant  $K = (HS)/(LS)$  is linear over the whole temperature range with an enthalpy of +10 kcal/mol.

Such a property was simultaneously found by Sligar<sup>14</sup> in a purely aqueous medium above 0 °C. The values of the thermodynamic parameters calculated by this author are  $K = 15$  at +20 °C and  $\Delta H = 2.47$  kcal/mol in phosphate buffer, pH 7. It must be noted that the HS/LS equilibrium is already shifted towards more HS by the progressive addition of ethylene glycol at room temperature, so that the 94% HS value calculated by Sligar becomes nearly 100% in the presence of 50% ethylene glycol at +20 °C. This value is chosen as a reference to calculate the HS/LS ratio at different sub-zero temperatures as well as the thermodynamic parameters of the equilibrium. The temperature range covered by the experiments provides a great amplitude of the changes and accurate conditions for precise measurements. The  $\Delta A_{417}$  values used for calculations have been corrected for the temperature variation of  $\epsilon_{417}$  measured on the free ferric cytochrome ( $m^0$ ) which is 100% low spin at sub-zero temperatures.

As shown in Table I, which summarizes the values of free energy, enthalpy and entropy changes,  $\Delta H$  increases when the protonic activity increases,

TABLE I

*Thermodynamic parameters of the LS  $\rightleftharpoons$  HS equilibrium of  $m^{0S}$  in 1:1 v/v cacodylate or phosphate buffer - ethylene glycol mixture*

$p_{a_H}$ at +20 °C	6	6.68	7.6
$\Delta H$ (kcal/mole)	+10	+8.2	+5.44
$\Delta G_{-20}$ °C (kcal/mole)	-1.44	-1.75	-1.76
$\Delta S$ (calK <sup>-1</sup> mol <sup>-1</sup> )	+45.2	+39.3	+28.4
$K =  HS / LS $			
- 10 °C	38.5	55.5	49.5
- 20 °C	17.6	32.4	33.5
- 30 °C	8.06	16.4	19.3
- 40 °C	3.8	8.4	13.3

indicating a proton dependent spin equilibrium. However the pH range within which the cytochrome is stable was not sufficient to evaluate the apparent pK of the transition.

A very interesting result emerging from the values in Table I is that the  $\Delta S$  variation with pH is opposite to the  $\Delta H$  variation, that is, there is a »linear compensation« of these terms with the  $\Delta G$  value almost constant when the pH changes. The compensation diagram shown in Figure 2 includes the present data and also the values obtained by Sligar above 0 °C in an aqueous medium<sup>14</sup>.

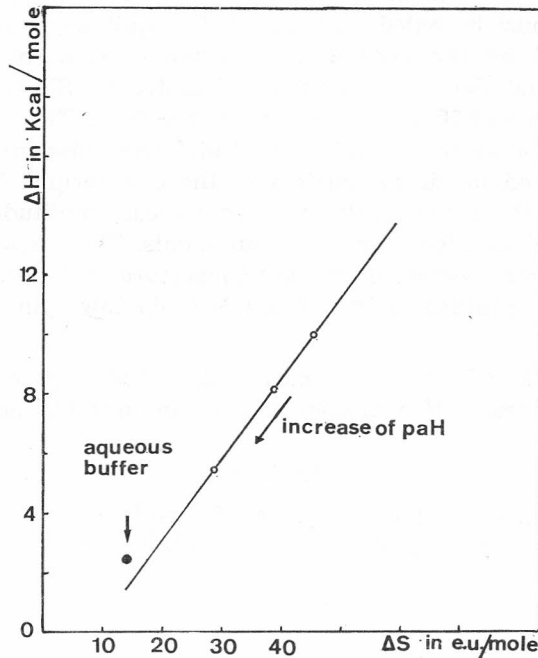


Figure 2. The plot of  $\Delta H$  versus  $\Delta S$  obtained for  $K = [\text{HS}]/[\text{LS}]$  in the subzero temperature range in a 1 : 1 v/v mixture of cacodylate or phosphate buffer and ethylene glycol (see Table I). The filled circle represents the values measured above 0 °C in aqueous buffer, pH 7 (Ref. 14).

### *Ethylisocyanide Compound of Ferrous Microsomal Cytochrome*

Another example of the striking features of cytochromes *P450* are the two different absorption bands (430 nm and 455 nm) which appear on the binding of lipophilic ligands to the reduced microsomal cytochrome *P450*, among which the most representative are the alkyl isocyanides<sup>15,16</sup>. Several interpretations have been proposed to explain this unique property, not exhibited by the bacterial cytochrome<sup>16,17</sup>. Recently various data have been collected by Peisach and fitted to the pH dependent equilibrium of species<sup>18</sup>. However some uncertainties concerning the reversibility of the pH-induced changes have remained unexplained. The addition of 50% (v/v) of EGOH shifts the 430/455 ratio towards 430 nm and a subsequent temperature analysis of the equilibrium shows that low temperatures favor the 430 nm form, an effect which is absolutely reversible (Figure 3).

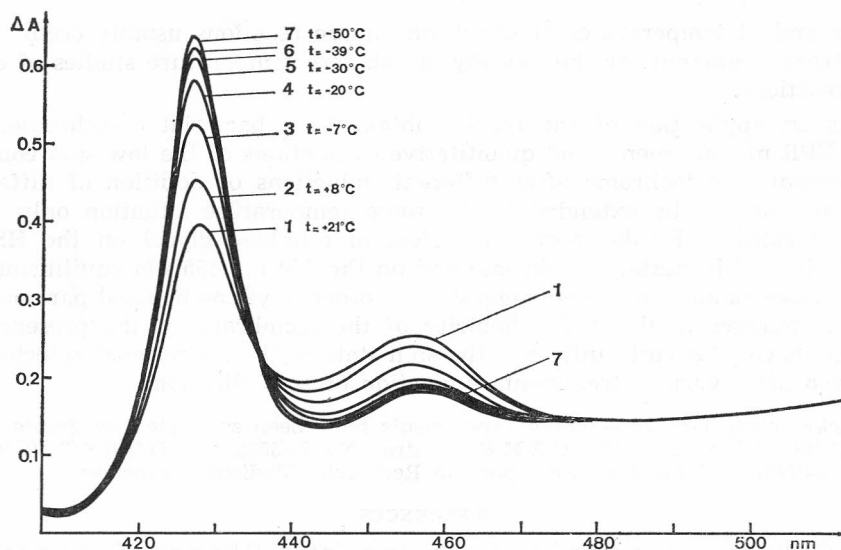


Figure 3. Temperature effect on the difference spectrum  $\text{Fe}^{2+} - \text{EtCN}$  minus  $\text{Fe}^{2+}$  of microsomal cytochrome P450. Microsomal suspension in a 3 : 7 v/v mixture of 0.02 M phosphate buffer pH 8 and glycerol. Ethylisocyanide  $5.10^{-4}$  M. Temperatures as indicated.

A change in the starting pH of the solution changed the initial 430/455 ratio without affecting the phenomenon qualitatively. It is very important to note the residual absorption at 455 nm which does not disappear even at very low temperatures and could be due to another type of cytochrome P450 not susceptible to equilibrium.

It seems rather unlikely that the changes observed could be due to changes in the membrane structure upon cooling, since no membrane transition was observed by other techniques in the same range of temperatures. Instead, these properties suggest that the 430/455 ratio is a real thermodynamic equilibrium between two energetically close conformations of the cytochrome. The nature of the difference between these two conformations is not yet understood. It may be due to the protonation of a group, changes in the geometry of the heme pocket, or movement, or even to the exchange of the 5th ligand<sup>18</sup>.

The earlier proposal of Peisach<sup>19</sup> that »the the unusual 455 nm chromophore may depend upon the dissociation of a proton from the sulfur-containing ligand« seems to agree with the recent results presented by Hanson *et al.*<sup>20</sup> These authors suggest that the abnormal 450 nm band of  $\text{Fe}^{2+} - \text{CO}$  arises from the mercaptide sulfur ligand, while the more normal  $\text{Fe}^{2+} - \text{O}_2$  compound may have a mercaptan sulfur ligand. The same distinction could apply to the 455 nm and 430 nm bands of the ethylisocyanide complexes. In such a case the addition of ethylene glycol and the lowering of temperature may increase the apparent pK value. A closer examination of the thermodynamic parameters of this transition may provide additional evidence for this hypothesis.

A major objective of this type of experimentation is to obtain the thermodynamic parameters of spin state and conformation equilibria over a wide temperature range in an homogeneous medium. Obviously there is a perfect continuity in the behavior of ferric cytochrome in a mixed solvent below zero

degree and at temperatures at which enzymatic reactions usually occur. This is a strong argument for the validity of sub-zero temperature studies of complex reactions.

As an application of the results obtained on bacterial cytochrome, the usual EPR measurements and quantitative evaluations of the low spin content of *microsomal* cytochrome after different inductions or addition of different substrates should be extended to the room temperature situation only with extreme caution. Furthermore, the effect of ethylene glycol on the HS/LS equilibrium of bacterial cytochrome and on the 430 nm/455 nm equilibrium of the ethylisocyanide compound suggests that other physico-chemical parameters, such as changes in the hydrophobicity of the membrane or the presence of detergents could greatly influence the spin state of the microsomal cytochrome observed after various treatments (induction or solubilization).

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## DISCUSSION

**D. L. Williams-Smith:**

If you freeze phosphate solutions large changes in pH of up to —3 pH units can occur, so there may be difficulties comparing data in liquid and solid states in case where phosphate buffers are used, such as optical data at —40 °C with EPR at 4.2 K.

**P. Debey:**

Yes, I agree completely with you.

**S. Vuk-Pavlović:**

Beetlestone and associates (Arch. Biochem. Biophys. **175** (1976) 138) have recently found that the spin state of aquomet derivative of haemoglobin is shifted towards the lower spin form in the presence of high concentrations of glycerol (above 95%). I was able to obtain a very similar optical spectrum in a 70% ethane diol — water solution of acid human methaemoglobin. Can you explain the shift of the spin equilibrium of the opposite sign in the solutions of m<sup>os</sup> upon addition of ethane diol? Could this effect be ascribed to an interaction between ethane diol and the protein since it seems that ethane diol is more effective as a solvent perturbant than glycerol?

**P. Debey:**

I do not know. Firstly, the heme ligands in the methaemoglobin and cytochrome P450 are different. Secondly, we do not know if this solvent effect is a *direct* one, through the entrance of a solvent molecule into the heme pocket, or an *indirect* one, that is, an effect on the whole protein which then affects the spin state. Thus, it seems difficult to compare directly different solvents and different hemoproteins.

**T. G. Traylor:**

Were you able to get from these or other pH studies the pK<sub>A</sub> of the possible group responsible for the transition to the low spin state?

**P. Debey:**

Not as yet, since for the moment we do not have a sufficient range of pH.

**R. H. Austin:**

Were you careful in avoiding the reducing properties of impure glycerol samples?

**P. Debey:**

Yes, we were. The same holds for ethylene glycol, which contains reducing impurities as well as some peroxides.

## SAŽETAK

**Miješana otapala za niske temperature u studiju strukturnih aspekata citokroma P450**

*R. Lange, P. Debey i P. Douzou*

Poznato je da su željezni ioni citokroma P450 različitog podrijetla smjesa vrsta s različitim elektronskim konfiguracijama koje stoje u djelomičnoj termodinamičkoj ravnoteži. Organska otapala kao etilenglikol ili glicerol, a i temperatura, djeluju izrazito, ali reverzibilno, na termodinamičke parametre tih ravnoteža u tekućem stanju. Perturbacija ravnoteže konformacijskih i spinskih stanja otapalom i temperaturom proučavana je za razna redoks-stanja citokroma P450 radi uvida u strukturu džepa hema.