The Mode of Binding of Carbon Monoxide to Iron in Cytochrome P450 and Cytochrome P420. An Infrared Study*

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The infrared stretching vibrations ($v_{CO}$) of the CO-complexes of cytochrome P450 and cytochrome P420 have been determined from the infrared difference spectra. The CO-complexes exhibit ir-bands at 1949 cm$^{-1}$ and 1966 cm$^{-1}$ with half widths of $\sim 17$ cm$^{-1}$ and $\sim 20$ cm$^{-1}$ respectively. These results are compared with the CO-stretching frequencies of other hemoproteins and discussed with respect to specific interactions of the CO-ligand with the protein moiety and to the ligand trans to CO in the cytochrome.

INTRODUCTION

The elucidation of the mechanism of activation of molecular oxygen by cytochrome P450** requires information concerning the binding properties of oxygen bound to the heme iron. Because of the instability of the oxygen-complex of P450 physical investigations have been done mostly with the carbon monoxide complex of P450.

The CO-complex is considered to be an appropriate model compound of the oxygen complex of P450 because carbon monoxide, as a ligand of the heme iron, reflects important characteristics of the linkage between oxygen and the heme iron.

From the absorption bands of the stretching vibration mode of the carbon monoxide complexes of hemoproteins in the infrared region information concerning the geometry and the binding type of the carbon monoxide ligand can be obtained\textsuperscript{1,3}.

The position, the half-width and the intensity of the CO stretching frequency are affected by cis and trans effects transferred by the heme iron. These parameters are also influenced by the direct interactions of the iron-bound CO with the protein moiety\textsuperscript{2}.

In an attempt to find out in what manner the different functional behaviours of hemoproteins can be correlated with differences in iron ligand binding properties as well as in ligand protein interactions, comparative infra-


** Abbreviations: P450 respectively P420 = the corresponding cytochromes; ir = infrared; Hb = hemoglobin; Mb = myoglobin.
red absorption studies of the stretching frequency of carbon monoxide complexes of cytochrome P450, of cytochrome P420 and of hemoglobin were undertaken.

EXPERIMENTAL

Infrared measurements were carried out with solubilized P450 partially purified by affinity chromatography from phenobarbital induced rabbit liver microsomes. The difficulties involved in the investigation of the CO stretching frequency of P450 are due to the relatively low concentration of the enzyme in the biological material. Therefore, we have concentrated the enzyme by ultrafiltration using Amicon membranes PM 30 to a concentration of about 0.5 mM. The P450 was converted into the CO-complex by the reduction of the heme iron with sodium dithionite and subsequent saturation of the solution with CO.

The infrared measurements were carried out using a Model 180 Perkin Elmer spectrometer operated with a resolution of 2 cm⁻¹ and with expanded ordinate and abscissa scales. The difference spectra were recorded by measuring the CO-complex against reduced hemoprotein solution in thermostated calcium fluoride cuvettes with 100 µm path length. The same cuvettes were used for the absorption measurements in the Soret region.

RESULTS AND DISCUSSION

Figure 1 shows an absorption band of the CO-complex of cytochrome P450 at 1949 ± 1 cm⁻¹ which in dependence on different times of incubation and on different temperatures is converted to a new one with an absorption maximum at 1966 ± 1 cm⁻¹. The conversion of the absorption band at 1949 cm⁻¹ is paralleled by the decrease in the absorption band of the CO-complex of cytochrome P450 in the Soret region. Furthermore, the appearance of the ir-band at 1966 cm⁻¹ is accompanied by an increase of the 420 nm band in the Soret

![Figure 1. Infrared difference spectra of the CO-complexes of P450 with different amounts of P420 (redrawn with corrected backgrounds); concentration of P450 = 4.4 · 10⁻⁴ M per heme; 6.1 M phosphate buffer, pH = 7.4; containing 30% (v/v) glycerol. ——— 10 min after preparation of the CO-complex, t = 18°C; ——— after 8 hours at 18°C; ———— after partial denaturation for 1 hour at 40°C; ———— after nearly complete denaturation for 3 hours at 40°C.](image)
CO BINDING TO CYTOCHROMES P450 AND P420, BY IR

region (Figure 2). There is an approximate linear relationship between the Soret intensity ratio (450 nm to 420 nm) and that for the corresponding infrared bands (1949 cm⁻¹ to 1966 cm⁻¹). Therefore, the infrared bands at 1949 cm⁻¹ and 1966 cm⁻¹ can be assigned to the CO-complexes of P450 and P420, respectively.

In a comparative analysis, the CO-complex of Hb was measured at the same concentration as that of the P450 solutions. This was necessary because the Hb · CO stretching frequencies have been usually measured at about one order of magnitude higher concentrations. On the other hand, with our material it was not possible to concentrate the P450 solution more than up to about 0.5 mM. The CO infrared band of the CO-complex of Hb in a concentration of 0.5 mM was found at 1951 cm⁻¹ with a half-width of 8.5 cm⁻¹ (see Table I). These data are the same as Alben and Caughey³ have reported for Hb · CO at higher concentrations.

Compared with the ir-band of P450 · CO that of Hb · CO shows an about twofold higher intensity, but roughly a two times smaller half-band-width. Therefore, we can estimate that the integrated intensities of both bands are nearly equal. An exact calculation of this correlation is under investigation.

The CO-complex of P450 with a half-width of 17 ± 2 cm⁻¹ exhibits the broadest half-width of all the CO-complexes of native hemoproteins studied so far and therefore differs significantly from Hb · CO as well as from Mb · CO. This striking result can be discussed by taking into account two possible explanations: 1. The band-broadening may be caused by the superposition of several absorption bands arising from different isoenzymes. 2. The broadening
**TABLE I**

Infrared data of CO-complexes

<table>
<thead>
<tr>
<th>CO-compound</th>
<th>CO-stretching frequency cm⁻¹</th>
<th>line width cm⁻¹</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450</td>
<td>1949</td>
<td>17±2*</td>
<td>4</td>
</tr>
<tr>
<td>Hb</td>
<td>1951</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Mb</td>
<td>1944</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>P420</td>
<td>1966</td>
<td>20±2</td>
<td>4</td>
</tr>
<tr>
<td>denatured Hb</td>
<td>1966</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>denatured Mb</td>
<td>1966</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>heme-mercaptide</td>
<td>1945</td>
<td>-</td>
<td>6</td>
</tr>
</tbody>
</table>

* Measurements of P450·CO in D₂O show a line width of 14±1 cm⁻¹

is caused by the interactions of the CO-ligand with charged groups originating from the external solvent or from the charged groups of the protein in the direct environment of the carbon monoxide.

The inactive form of P450, *i.e.* P420, corresponds to denatured hemoproteins. Indeed, the broad half-width of the CO infrared band of P420 with 20 ± 2 cm⁻¹ is equal to the half-width of CO infrared bands of acid denatured Hb · CO and Mb · CO. According to Caughey *et al.*² the larger half-width of the CO infrared bands of denatured Hb · CO and Mb · CO compared with their native forms might be due to the fact that in the denatured state interactions of the heme iron bound CO with the external solvent predominate over specific interactions of the CO ligand with the protein. This conclusion is supported by the fact that the CO infrared bands of Hb · CO and Mb · CO have the same line width.

Moreover, the fact that the half-width of the CO infrared band of P420 equals that of denaturerated Hb · CO and Mb · CO is a strong evidence for the assumption that the CO-ligand is in good contact with the external solvent also in P420 · CO. This assumption is furthermore supported by the correspondence of the stretching frequencies: 1966 cm⁻¹ for the P420 · CO and for denatured Hb · CO and Mb · CO, as well. The same position of the CO infrared bands of denatured Hb · CO and Mb · CO indicates that in the denatured state specific interactions of the CO-ligand with amino acid residues of the heme pocket are obviously abolished. The linkage between the iron and the histidine trans to CO is, however, retained as Caughey *et al.*² have shown by a comparison with model complexes. This means that in the dena-
tured state of hemoglobin and myoglobin the ligand trans to CO mainly determines the position of the CO infrared band. Therefore, it can be concluded that the same spectral properties of the CO infrared band of \( P420 \cdot CO \) and denatured \( Hb \cdot CO \) and \( Mb \cdot CO \) do not only indicate a good contact of the solvent with CO in \( P420 \cdot CO \) but also that the ligand trans to CO is probably an imidazole.

On the other hand, it has been proposed that in the native form of \( P450 \) the fifth ligand is a mercaptide. In the CO complex of \( P450 \) this would be the ligand trans to CO. Furthermore, it can be assumed that the \( \pi \)-donor properties of the mercaptide account for the unusual position of the Soret band. From these properties of the assumed fifth ligand of \( P540 \) and from the influence of the trans ligand on the CO stretching frequency a great difference between the CO stretching frequencies of \( P450 \cdot CO \) and \( Hb \cdot CO \) may be expected. Instead of this, the infrared bands of the CO-complexes show an unexpectedly small difference. In order to explain this behaviour it is necessary to take further hemoproteins into account. The CO stretching frequency of \( Mb \cdot CO \) in which, as in Hb, the ligand trans to CO is an imidazole is found at 1944 cm\(^{-1}\). This comparison obviously indicates that it is questionable to draw conclusions from the CO-stretching frequency in native hemoproteins as to the nature of their ligands in trans position. The position of the CO stretching frequencies of CO-compounds of hemoproteins are rather determined not only by trans effects but also by cis effects and direct interactions between the amino acid residues in the heme pocket with the CO ligand, existing as van der Waals and charge transfer interactions. Therefore, it is not surprising that the CO-stretching frequency of \( P450 \) does not agree with the model complex of \( P450 \) measured by Collman et al. The stretching frequency 1945 cm\(^{-1}\) of this heme complex, with a mercaptide trans to CO, is much more similar to the stretching frequency of \( Mb \cdot CO \). Therefore, we cannot determine the nature of the trans ligand of \( P450 \cdot CO \) by the stretching frequency of this model complex. Moreover, the great difference between the CO-frequencies of the native \( P450 \) and those of the model complex is additional, strong evidence that the CO stretching frequency of native \( P450 \) is determined by specific interactions of the heme \( \cdot CO \) complex with the protein and not by unspecific interactions with the solvent.

Since model complexes do not reflect the real structure of \( P450 \cdot CO \), especially not the important interactions of the iron-bound CO with the protein moiety, further investigations of the enzyme under different conditions especially in the presence of substrates, are necessary to get an insight into the real structure. Such investigations are now under way.

REFERENCES

**DISCUSSION**

T. G. Traylor:
Our imidazole model carbon monoxide complex has a stretching frequency of 1964 cm⁻¹ in solution and ~ 1950 cm⁻¹ as a solid. This is in agreement with your conclusion concerning distal side interactions. How do you explain the observation that CO models of hemoglobin, presumably having no distal side interference, absorb at 15—18 cm⁻¹ higher frequencies whereas the thiolate CO model absorbs at a 5 cm⁻¹ lower frequency than your P450-CO complex?

H. Rein:
If there is no distal side interference of CO then the trans ligand determines mainly the CO stretching frequency. The lower frequency of the thiolate CO model complex may be caused by the better π-donor properties of the mercaptide compared to the imidazole. These properties of the mercaptide may lead to a weakening of the π-binding of CO and therefore we observe the shift to lower frequencies.

H. Schleyer:
How did you make your » P420 « — and if you made it by different methods — how do they differ?

H. Rein:
We produced our P420 in two different ways:
1. Thermal denaturation; if the P450 sample is kept for about 3 hours at 40 °C most of the enzyme is converted into P420 (cf. Figure 2 of the paper, — ).
2. Addition of sodium deoxycholate; pure P420-CO produced in this way shows the same characteristics of the infrared band as the P420 obtained by heating.

**SAŽETAK**

Veza ugljičnog monoksida sa željezom u citokromima P450 i P420 prema infracrvenoj spektroskopiji

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Vibracije rastezanja u CO-kompleksima odredene su iz diferencijalnih infracrvenih spektara za oba citokroma: 1949 cm⁻¹ sa širinom vrpce 17 cm⁻¹ za P450, i 1966 cm⁻¹ sa širinom vrpce 20 cm⁻¹ za P420. Ti se rezultati uspoređuju sa sličnim podacima za druge hemoproteine i modelne spojeve analizirajući specifične interakcije CO-liganda s proteinom i u odnosu na ligand u trans-polofaju prema CO u citokromu.

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