Activation Volume and Energetic Properties of the Binding of CO to Hemoproteins

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ABSTRACT We have investigated the CO binding to various reduced hemoproteins by stopped-flow rapid mixing as a function of pressure (from 0.1 to 200 MPa) and temperature (from 4 to 35°C). In particular, we studied several varieties of cytochrome P-450: CYP11A1 (scc), CYP2B4 (LM2), CYP3A6 (LM3c), and Cyp2a (7α), as well as chloroperoxidase and lactoperoxidase, and compared the results to data reported for other hemoproteins. Whereas the CO binding activation enthalpy $\Delta H^{\ddagger}$ and entropy $\Delta S^{\ddagger}$ (correlated through a compensation effect) varied greatly between the hemoproteins, with no apparent relation to structural features, the pressure effect depended on the nature of the proximal axial heme ligand: the activation volume was very small for cysteine (S^-) ligand hemoproteins ($\Delta V^{\ddagger} = +1$ to $+6$ ml mol^{-1}), and markedly negative for histidine (N) ligand hemoproteins ($\Delta V^{\ddagger} = -3$ to -36 ml mol^{-1}). Furthermore, the transition state volume of the histidine ligand class enzymes, but not that of the cysteine ligand enzymes, depended on the solvent composition. These results suggest that the CO-binding transition state of the S-ligand class has a molecular conformation similar to the ground state. In the histidine class, however, the transition state appears to involve protein conformational changes and/or solvation processes.

INTRODUCTION

The dynamic nature of proteins is established by an overwhelming wealth of experimental evidence, including spectroscopic studies such as NMR, EPR, and fluorescence, as well as from analogies with model systems and from molecular dynamics simulation studies (Karplus and McCammon, 1981; Chou, 1983; Robach et al., 1983; Wagner, 1983; Henry et al., 1986; Nienhaus et al., 1992). Even in the resting state, enzymes fluctuate between different conformational states. According to the pioneering work of Frauenfelder et al. (1991), these conformational states appear to be organized hierarchically in substates (Ehrenstein et al., 1991; Steinbach et al., 1992). The fluctuations between these states may occur on a large scale or be restricted locally. Furthermore, the enzyme reaction, which may involve one or a combination of several of these fluctuations, often depends on the specific interaction with the surrounding solvent. Our understanding of the underlying laws and principles of protein dynamics is however still in its infancy; clearly, the classical transition state theory (TST) (Eyring, 1935) with its mechanistic approach ($E + S \rightarrow ES^{\ddagger} \rightarrow ES$) is an oversimplification of reactions carried out by complex macromolecules like proteins. Furthermore, the key piece of this theory—the transition state—is the most poorly defined (often we know only its relative enthalpy and entropy). Nevertheless, in the absence of a better-adapted and generally accepted theory, we continue here to use the TST formalism. But clearly, in order to modify or to adapt the TST to the complexity of enzyme reactions, the transition state must be characterized structurally.

Our method is high pressure stopped-flow mixing (Heremans et al., 1980) operating at different temperatures, which we developed some years ago, and have applied since to the study of several enzyme systems (Balny et al., 1984, 1987; Saldana and Balny, 1992; Heiber-Langer et al., 1992). This method allows one to determine the activation volume of a reaction (that is, the volume change of the transition state with respect to the ground state) from the dependence of the rate constant, k, on pressure (up to 200 MPa) according to

$$\partial \ln k/\partial p = -\Delta V^{\ddagger}/RT. \quad (1)$$

By varying both pressure and temperature, one is able to obtain simultaneously kinetic (k), energetic ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$) and structural ($\Delta V^{\ddagger}$) information on a reactional transition state. For example, the magnitude of the activation volume $\Delta V^{\ddagger}$ can result from conformational changes in major or restricted regions of the protein. It can also reflect a peculiar interaction of the transition state with solvent molecules. Furthermore, the sign of $\Delta V^{\ddagger}$ can be positive or negative, depending on the type of the chemical reaction (Morild, 1981; Balny et al., 1985, 1989).

Our enzyme model is the binding of carbon monooxide to ferrous hemoproteins with a special emphasis on cytochrome P-450. This reaction is an example of the interaction of a protein with a small uncharged ligand. The reaction can be followed easily by recording the absorbance change in the Soret band, where the spectral change is particularly large. Furthermore, the CO binding may be used as a model for a better understanding of the interaction of cytochrome P-450 with oxygen. The activation of oxygen is, of course, the key

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TABLE 1 Experimental conditions for the CO binding of different hemoproteins

Enzyme	Concentration of			Buffer	pH	$\lambda_1 - \lambda_2$		t
	Enzyme	Dithionite	CO					
	μM	mM	μM			nm		°C
$P\text{-}450_{scc}$	3.4	2.1	50	MOPS (10 mM)	7.0	448.5	406.5	5.2–25.0
P-450LM2	3.4	4.2	5	Phosphate (50 mM), 8.1% glycerol	6.8	451.5	407	4.0–14.5
P-450LM3	0.84	3.6	3	Phosphate (50 mM), 20% glycerol, 0.1 mM EDTA	7.4	449.5	406.5	6.0–25.0
P-4507α	1.1	6.0	5	Phosphate (100 mM), 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.4% cholate, 0.2% emulgen, 100 μM testosterone	7.25	449.5	406.5	5.0–25.0
CPO	3.2	2.1	5	Citrate (10 mM)	4.5	446	405	5–25.0
LP	3.6	2.1	475	MOPS (10 mM)	7.0	426	404.5	25.0
P-420	3.4	2.1	50	Phosphate (50 mM)	7.4	420	438	25.0

All concentrations are final concentrations after mixing in the stopped-flow apparatus. CPO = chloroperoxidase and LP = lactoperoxidase.

step in the substrate hydroxylation catalysed by cytochrome P-450 (White and Coon, 1980). A wealth of experimental evidence indicates that O_2 activation by cytochrome P-450 depends on the presence of thiolate as the fifth proximal heme-iron ligand (Rein et al., 1984). It was therefore important to know the way in which the thiolate ligand affects the transition state of the CO binding reaction. For this purpose, we compared the results obtained with thiolate ligated hemoproteins (various cytochromes P-450 and chloroperoxidase) with those obtained with histidine ligated hemoproteins (lactoperoxidase, horseradish peroxidase (HRP), hemoglobin, myoglobin, and heme P-460, a part of the active site of the multiheme enzyme hydroxylamine oxidoreductase, from *Nitrosomas europaea* (Arciero et al., 1993). The identity of the fifth heme ligand of chloroperoxidase as a sulfur is well established now by optical absorption, electron spin resonance, Mößbauer properties and resonance Raman spectra (Campbell et al., 1982). Furthermore, we analyzed the dependence of the transition state volume on the solvent polarity and viscosity and on physiological effector molecules.

The results suggest discrete strategies which different hemoproteins can adopt to form reactional transition states.

MATERIALS AND METHODS

Cytochrome P-450 scc (CYP11A1)[1] was isolated from bovine adrenocortical mitochondria using previously described methods (Lange et al., 1988). Cytochrome P-450 LM2 (CYP2B4) was isolated from rabbits which had been under phenobarbital treatment for one week according to Coon et al. (1978). Cytochrome P-450 LM3c (CYP3A6) was induced in rabbits by rifampicine and isolated as described (Lange et al., 1985). These isolated enzymes were purified to electrophoretic homogeneity. Cytochrome P-450 7α (Cyp2a) was isolated from yeast as the gene expression product of the corresponding cDNA according to Iwasaki et al. (1993). The isolated P-450 7α fraction was avoid of any other P-450 form. In order to prevent its conversion to P-420, testosterone (100 μM) was added to the buffer. Cytochrome P-420 was produced by the dilution of concentrated cytochrome $P\text{-}450_{scc}$ into phosphate solution (50 mM, pH 7.4) containing 5% (v/v) butanol. The cytochrome P-420 formed in this way was used immediately. Lactoperoxidase was from Calbiochem (San Diego, CA) and chloroperoxidase from Sigma Chemical Co. (St. Louis, MO).

All cytochrome P-450 stock solutions were stored at −80°C in 20% glycerol as a protective agent. For the cytochromes P-450 and for lactoperoxidase we used either 4-morpholine propanesulfonic acid (MOPS) buffer the pK of which is almost pressure-independent (Neumann et al., 1973; Kitamura and Itoh, 1987) or phosphate buffer. The results did not depend on the buffer composition. For chloroperoxidase we used citrate buffer. Although the pH of this buffer decreases up to 0.6 pH units at high pressures (>100 MPa) (Lambeir et al., 1983), the CO-binding reaction is not expected to be affected significantly by such a pH change. In fact, the binding of CO to chloroperoxidase depends on an ionizable group with pK = 5.5 (Campbell et al., 1982). The enzyme solutions (2–6 μM) were deoxygenated by blowing argon upon their surface. The enzymes were completely reduced by reaction with sodium dithionite (2–6 mM final concentration) under argon for 30 min.

A CO stock solution was prepared by bubbling oxygen-free argon for 30 min through the buffer solution and then flushing with CO for 45 min. The concentration of this stock solution was taken to be 1 mM CO at 20°C at atmospheric pressure. CO solutions were then prepared by diluting this stock solution with oxygen-free buffer.

Kinetic measurements at both atmospheric and high pressure (up to 200 MPa) were performed in stopped-flow devices developed in Montpellier (Markley et al., 1981; Balny et al., 1984) interfaced to Aminco DW2 spectrophotometers. We always mixed equal volumes of an enzyme solution with the CO solution. The buffer composition and final glycerol content was identical in both solutions. All kinetics were recorded in the dual-wave length mode of the Aminco using the wavelengths of the maximum, λ_1, and minimum, λ_2, absorption of the CO complex. Details on buffer composition and wavelengths are given in Table 1.

Data were stored by a metrabyte A/D converter board and a personal computer and were analyzed using a Marquardt fit. The mono- and biexponential kinetics were fitted to the following equations:

$$A_t = A_1 \cdot \exp(-k_{obs_1} \cdot t) + A_\infty \quad (2)$$

$$A_t = A_1 \cdot \exp(-k_{obs_1} \cdot t) + A_2 \cdot \exp(-k_{obs_2} \cdot t) + A_\infty \quad (3)$$

with A_t = absorption at time t, A_1 and A_2 the amplitudes of both effects, k_{obs_1} and k_{obs_2} the observed rate constants and A_∞ the end absorption. The quality of the fit was evaluated by a χ^2 analysis and by a plot of the residuals (see for an example the inserts of Figs. 2 and 4). All experiments were run at least three times.

[1] *Abbreviations used:* CPO, chloroperoxidase; HRP, horseradish peroxidase; Hb, hemoglobin; mb, myoglobin; LP, lactoperoxidase. For the various cytochrome P-450 enzymes we used the new nomenclature recommended by Nelson et al. (1993).

RESULTS

Pressure and temperature effect on CO binding to hemoproteins

In order to evaluate the pressure effect on the CO binding reaction, we established first reference data under standard conditions, that is under atmospheric pressure and (when possible) at 25°C. Second, we had to work out the dependence of the observed rate constants, k_{obs}, on the CO concentration. Whenever this determination was experimentally possible, we found a linear relationship between k_{obs} and the CO concentration. Therefore, we described the kinetic data by the simple one step model

$$E + CO \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}CO$$

SCHEME 1

where

$$k_{obs} = k_1[CO] + k_{-1}. \quad (4)$$

For all enzymes, ln k_{obs} varied linearly as a function of the inverse temperature, $1/T$, and of the pressure, p. The determinations of $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta V^{\ddagger}$ were therefore straightforward. Since k_{-1} was always much smaller than k_1—as shown below, the dissociation constant $K_d = k_{-1}/k_1$ was in the micromolar range—the thermodynamic activation parameters we measured for the various enzymes characterise the binding rate, k_1. In Table 2, these results are compared for the various hemoproteins.

CYP11A1 (P-450scc)

We observed only one relaxation effect. As shown in Fig. 1, the observed rate constant k_{obs} increased linearly as a function of the CO concentration. We determined the second order rate constants as $k_1 = 2.3 \times 10^5 \pm 0.1$ M^{-1} s^{-1} and

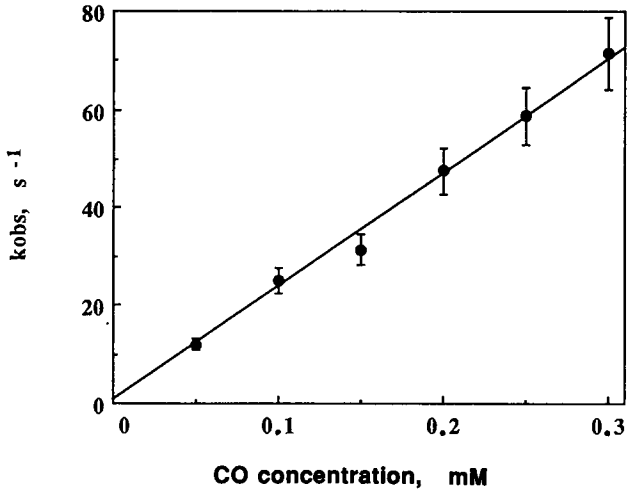

FIGURE 1 CO binding to cytochrome P-450_{scc}. Correlation of the observed rate constant k_{obs} with the CO concentration; the concentration of cytochrome P-450_{scc} was 3.4 μM; t = 25°C, p = 0.1 MPa.

$k_{-1} = 0.063 \pm 0.05$ s^{-1}. The value of k_1 is consistent with the rate constant determined by Tuckey and Kamin (1983), 2.2×10^5 M^{-1} s^{-1}, and by Kashem et al. (1987), 1.9×10^5 M^{-1} s^{-1}. The value of k_{-1} is more difficult to determine, as it is very small, and its determination may depend on the experimental CO concentration range: for a comparison, Tuckey obtained 0.15 s^{-1} and Kashem 12 s^{-1}. The pressure effect on k_1 was very small: we determined a value of $\Delta V^{\ddagger} = 2 \pm 2$ ml mol^{-1}. Between 5 and 25°C, the temperature did not affect the activation volume. Up to 150 MPa cytochrome P-450_{scc} was stable, and we did not observe a notable conversion to P-420 (see also Fig. 7).

CYP2B4 (P-450 LM2)

For this enzyme we observed two relaxation effects. The first one was at least 10 times faster than the second one,

TABLE 2 Activation thermodynamic parameters of the CO binding to various hemoproteins

Enzyme	Ligand	k_1 (25°C, 0.1 MPa)	$\Delta V^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	Reference
		M^{-1} s^{-1}	ml mol^{-1}	kJ mol^{-1}	J K^{-1} mol^{-2} L	
P-450_{scc}	S^- (cys)	2.3×10^5	2 ± 2	25 ± 4	-58 ± 12	This work
P-450LM2	S^- (cys)	$3.1 \times 10^{6*}$	3 ± 2	34 ± 4	-9 ± 10	id.
P-450LM3	S^- (cys)	2.2×10^6	6 ± 5	41 ± 4	$+16 \pm 10$	id.
P-4507α	S^- (cys)	$2.8 \times 10^{4*}$	2 ± 2	18 ± 2	-99 ± 10	id.
CPO	S^- (cys)	1.4×10^5	1 ± 2	26 ± 3	-59 ± 10	id.
LP	N (his)	8.0×10^2	-10 ± 3	n.d.	n.d.	id.
HRP	N (his)	2.8×10^3	-24	41.7	-37	Balny (1989)
Myoglobin	N (his)	3.8×10^5	-9	17	-81	Hasinoff (1974)
			-18.8			Adachi (1989)
Hemoglobin	N (his)	2.8×10^6	-3	23	-41	Hasinoff (1974)
		6.0×10^4	-21	41	-12	id.
P-460	N (his)	2.8×10^3	-36	35	-60 ± 20	Balny (1988)
P-420	Unknown	$66 \times 10^{3\dagger}$	-25 ± 5	78 ± 3	$+26 \pm 11$	This work
		$12 \times 10^{3\dagger}$	-11 ± 6	97 ± 6	$+76 \pm 20$	id.
		$0.96 \times 10^{3\dagger}$	-42 ± 8	n.d.	n.d.	id.

* Determined from the Arrhenius plot.

† Phases 1 to 3; n.d., not determined; HRP, horse radish peroxidase.

$\Delta S^{\ddagger}$ was determined from the Arrhenius preexponential factor A according to $\Delta S^{\ddagger} = R\{\ln(A{\cdot}h/k_BT) - 1\}$.

and its spectral amplitude was more than 90% of the total amplitude. We analyzed therefore only the fast effect. The origin of the second phase is not clear: a contamination by another P-450 form is unlikely, since our preparation was very pure. Perhaps it is related to a heterogeneity in the membrane environments. As in the case of cytochrome P-450$_{scc}$, k_{obs} depended linearly on the CO concentration. We could verify this linear relationship from 5 to 100 μM CO at 4°C. At higher temperatures and at higher CO concentration the reaction was too fast for our stopped-flow apparatus. Using Eq. 4 we determined the second order rate constants as $k_1 = 9.1 \times 10^5 \pm 1$ M^{-1} s^{-1} and $k_{-1} = 6 \pm 3$ s^{-1}. Very similar results had been obtained by a hemoglobin/P-450 CO exchange method (Balny, and Debey, 1976). The k_1 value was two to three times higher than that obtained by flash-photolysis (Omura et al., 1965; Bonfils et al., 1979). Our results are in a reasonable agreement with the extensive stopped-flow investigation of Gray (1982). For 25°C, he determined a second order rate constant $k_1 = 2$–4×10^6 M^{-1} s^{-1} and a back-reaction constant of $k_{-1} = 20$ s^{-1}. Our lower values of k_1 and k_{-1} are explained by the activation enthalpy of $\Delta H^{\ddagger} = 34$ kJ mol^{-1} (see below). In contrast to our results, Gray reported a hyperbolic function of k_{obs} versus the CO concentration, and from that he deduced a reactional scheme corresponding to the induced fit model. His published experimental data offer however the possibility of other interpretations: in fact a closer analysis shows that his $k_{obs} = f([CO])$ values for LM2 can be fitted very well by a straight line. Another difference is that Gray observed two additional kinetic phases. One may speculate that these additional phases are due to contaminations from other cytochrome P-450 forms; our preparation was electrophoretically pure.

Similarly to P-450$_{scc}$, the activation volume of k_1 was small: $\Delta V^{\ddagger} = 1.5 \pm 3$ ml mol^{-1}, and temperature independent between 4 and 14.5°C.

CYP3A6 (P-450 LM3c)

This cytochrome P-450 is different from other P-450 enzymes with respect to the mechanism of oxygen binding to the ferrous form: even at low temperature (−40°C) an oxy-compound is not detectable (unpublished results). It was therefore interesting to study its CO binding mechanism. The CO binding of LM3c was biphasic with the slow phase representing about 15% of the amplitude of the fast effect. The relaxation time for the slow effect was greater than 10 s. For the fast effect a second order rate constant could be calculated at 25°C as $k_1 = 2.2 \times 10^6$ M^{-1} s^{-1}. The activation volume was determined as $\Delta V^{\ddagger} = 6 \pm 5$ ml mol^{-1}.

Cyp2a (P-450 7α)

This cytochrome is unstable in its reduced form: some minutes after reduction, CO binding results in the formation of cytochrome P-420. The formation of P-420 could be avoided by carrying out the experiments in the presence of its substrate (testosterone) (Iwasaki et al., 1993). The CO-binding of 7α was monoexponential and much slower than that of other cytochromes P-450s. We observed a linear dependence of k_{obs} as a function of the CO concentration up to the maximally attainable concentration (0.5 mM). From the straight line the forward and backward rate constants were calculated at 25°C as $k_1 = 1.6 \times 10^4$ M^{-1} s^{-1} and $k_{-1} = 0.7$ s^{-1}. The activation volume related to k_1 at 25°C was $\Delta V^{\ddagger} = 2 \pm 2$ ml mol^{-1}.

Chloroperoxidase

This peroxidase was chosen, because of the similarity of its heme environment to cytochromes P-450. As shown in Fig.2, the CO binding was monophasic. From the linear dependence of k_{obs} on the CO concentration we determined the pseudo first order binding rate constant as $k_1 = 6 \times 10^4$ M^{-1} s^{-1} and the off rate as $k_{-1} = 2$ s^{-1} at 25°C. These values were in agreement with those obtained by Campbell et al. (1982). Fig. 2 shows a typical CO binding experiment at elevated pressure (130 MPa). The pressure dependence of k_1 was determined at 25°C as $\Delta V^{\ddagger} = 1 \pm 2$ ml mol^{-1}. It did not vary significantly as a function of temperature.

Lactoperoxidase

For this enzyme we observed biphasic CO binding kinetics. Both phases had about the same amplitude. The slow phase was a factor of 10 slower than the fast one. As the slow reaction was too slow for the stopped-flow apparatus (τ = 30 s) even at saturation of CO, we did not measure the CO dependence of k_{obs}. The second order rate constants were then calculated from the k_{obs} values at CO = 475 μM as $k_1 = 8 \times 10^2$ M^{-1} s^{-1} for the fast effect and $k_2 = 75$ M^{-1} s^{-1} for the slow effect. As shown in Fig. 3, the slope of ln $k_1 = f(p)$ was opposite to that obtained with cytochrome P-450$_{scc}$. At 25°C we determined a value of $\Delta V^{\ddagger} = -10 \pm 3$ ml mol^{-1}.

Cytochrome P-420

As shown in Fig. 4, the kinetics could be fitted by three exponentials. The same fit could be applied under the various experimental conditions, and the plot of the residuals (see insert) was always satisfactory. Other fitting models, such as only two exponentials or one stretched exponential of the form $A_t = A_1 \exp[-(k \cdot t)^{\beta}] + A_{\infty}$, as proposed by Frauenfelder et al. (1991) for reactions comprising a multiplicity of protein conformations, were not satisfactory. At 25°C, the second order rate constants (calculated from the k_{obs} values obtained with a CO concentration of 50 μM) were: $k_1 = (66 \pm 6)\ 10^3$ M^{-1} s^{-1}, $k_2 = (12 \pm 1)\ 10^3$ M^{-1} s^{-1} and $k_3 = (0.96 \pm 0.14)\ 10^3$ M^{-1} s^{-1}. The corresponding spectral amplitudes were $A_1 = 50\%$, $A_2 = 30\%$, and $A_3 = 20\%$. Cytochrome P-420 was more sensitive to pressure than cytochrome P-450: the solution became slowly turbid at pressures higher than 50 MPa. Nevertheless, in the 0.1 to 50-MPa pressure range, the plots of ln $k = f(p)$ were linear, and we could

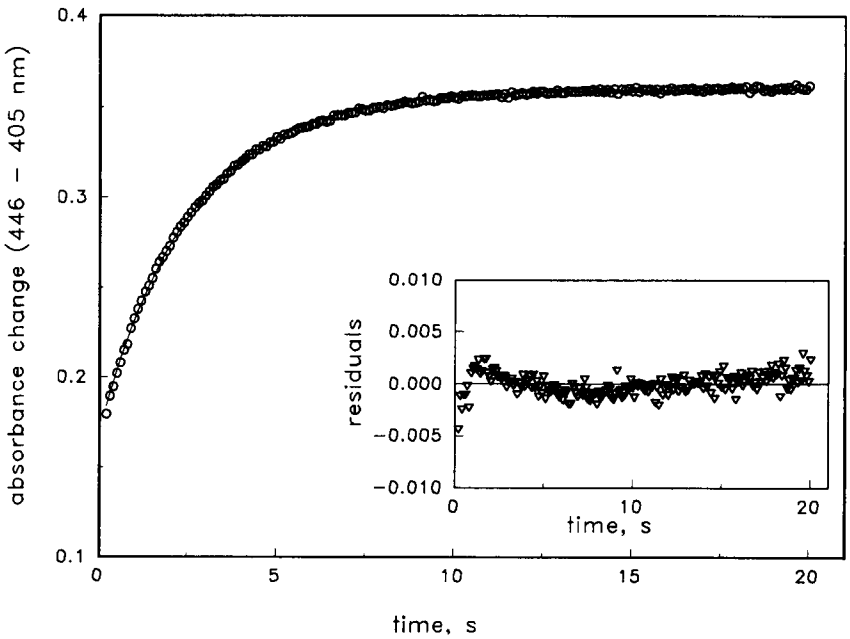

FIGURE 2 Time course of the CO binding to chloroperoxidase after stopped-flow mixing at high pressure. [Chloroperoxidase] = 3.2 μM, [CO] = 5 μM; t = 5°C, p = 130 MPa; the mono-exponential fit is shown by the solid line through the data points (*circles*). The insert shows the residuals (data points - fit).

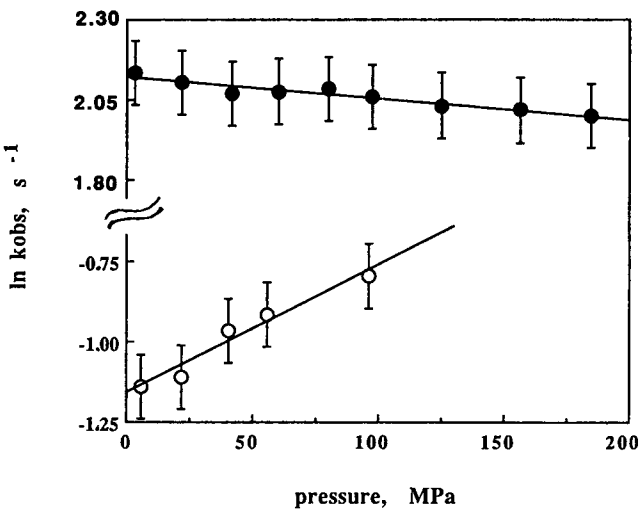

FIGURE 3 Pressure dependence of the CO binding rate k_{obs}. Full circles, cytochrome P-450$_{scc}$; open circles, lactoperoxidase.

determine the activation volumes related to the three rate constants as $\Delta V_1^{\ddagger} = -25 \pm 5$ ml mol^{-1}, $\Delta V_2^{\ddagger} = -11 \pm 6$ ml mol^{-1}, and $\Delta V_3^{\ddagger} = -42 \pm 8$ ml mol^{-1}.

The above results are summarized in Table 2. Clearly, the hemoproteins fall in two classes, depending on the nature of their proximal axial heme iron ligand. The sulfur-ligand hemoproteins, cytochromes P-450 and chloroperoxidase, are characterized by small positive activation volumes: $\Delta V^{\ddagger}$ = 1 to 6 ml mol^{-1}. The other class, the nitrogen-ligand hemoproteins (hemoglobin, myoglobin, horseradish peroxidase, and lactoperoxidase) are characterized by markedly negative activation volumes: $\Delta V^{\ddagger} = -3$ to -36 ml mol^{-1}. An example for both classes is given in Fig. 3, showing the pressure dependence of lactoperoxidase ($\Delta V^{\ddagger} = -10$ ml mol^{-1}) and P-450$_{scc}$ ($\Delta V^{\ddagger} = +2$ ml mol^{-1}). We have verified for several enzymes that the temperature did not affect significantly the activation volume. Other parameters, such as the bimolecular CO binding rate constant k_1, the activation enthalpy $\Delta H^{\ddagger}$ and the activation entropy $\Delta S^{\ddagger}$ differed considerably from one enzyme to another, without distinction between the S- and the N-ligand hemoproteins. Cytochrome P-420 may belong to yet a third class: its activation volume was negative, as in the case of the N-ligand hemoproteins, but its $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values were considerably greater than those of the hemoproteins of the two other classes.

Solvent effect on the CO binding of P-450scc

The CO binding reaction of cytochrome P-450$_{scc}$ appears to be mostly solvent-independent. As shown in Table 3, we have varied the temperature (from 25 to 5°C), the pH (from 7 to 8.2), the ionic strength (up to 300 mM NaCl), the polarity of the solvent (water, 40% ethylene glycol and 50% glycerol), and the solvent viscosity (η/η_0 = 1–6). In none of these conditions was the activation volume affected. The binding rate constant remained constant as well, except in 50% glycerol, where it was lowered by a factor of 10.

DISCUSSION

Kinetic model

Since we observed in all cases a linear relationship between k_{obs} and the CO concentration, we interpreted the kinetic data in terms of the one-step binding model where $k_{obs} = k_1[CO] + k_{-1}$. It is however worthwhile, to consider another model, which has been used by Balny and Travers (1989), as well as by Gray (1982), for the interaction of CO with hemoproteins. That model is based on the general induced fit theory, and it supposes a two step process: a first rapid formation of an enzyme-CO adduct

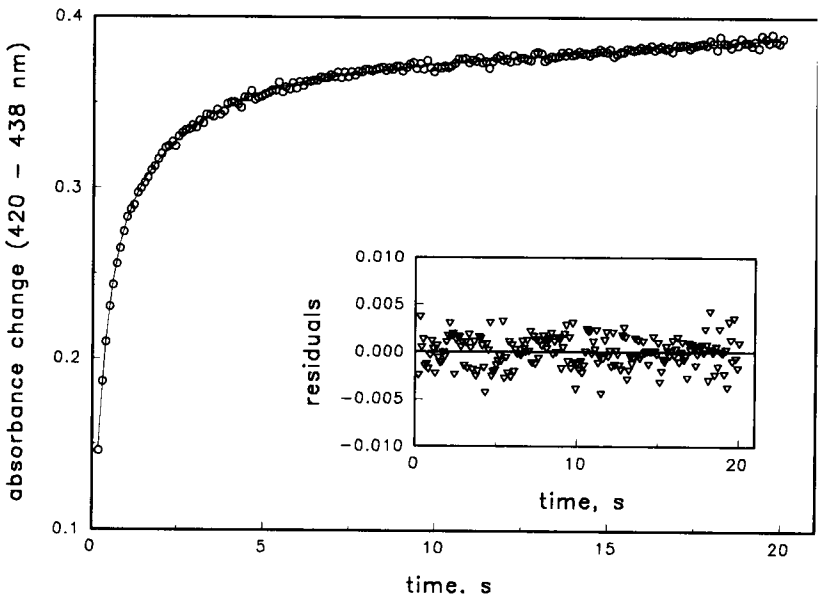

FIGURE 4 Kinetics of the CO binding to cytochrome P-420. Cytochrome P-450$_{scc}$ was converted to P-420 by addition of butanol (5%, v/v) to the buffer.

TABLE 3 Solvent dependence on the P-450scc CO binding rate constant k_1 and on its activation volume $\Delta V^{\ddagger}$

Experimental condition	k_1	$\Delta V_{\ddagger}$	η/η_0
	$M^{-1}\ s^{-1}$	ml mol^{-1}	
Aqueous MOPS buffer, pH 7			
at 25°C	2.1×10^5	2 ± 2	1
at 5°C		2 ± 2	1
Tris buffer, pH 8.2	2.0×10^5	1 ± 1	1
300 mM NaCl	2.3×10^5	0 ± 1	1.03
40% ethylene glycol	2.4×10^5	5 ± 4	2.8
50% glycerol	2.8×10^4	0 ± 2	6.0

The bimolecular rate constant was calculated from the observed rate constant at a CO concentration of 50 μM. The solvent composition is to be understood as a modification of the standard condition (aqueous MOPS buffer, pH 7, at 25°C).

complex, followed then by an isomerization step:

$$E + CO \underset{}{\overset{K_d}{\rightleftharpoons}} E \cdot CO \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^* \cdot CO$$

SCHEME 2

where $K_d = k_{-1}/k_1$. As pointed out by Hiromi (1979) and Gray (1982), this model leads to two relaxation steps, a fast, diffusion-controlled step, with $k_{obs_1} = k_1[CO] + k_{-1}$, and a slower step, with $k_{obs_2} = k_2[CO]/(K_d[CO])$. Whereas k_{obs_1} is too fast to be measured, k_{obs_2} would reach a plateau at high [CO] concentration. Now, since we are limited in the maximal CO concentration to 0.5 mM, it may be, that our measurements are within a first linear part of $k_{obs_2} = f([CO])$. With that model, the initial slope, s_i, becomes $s_i = (k_2/k_{-1})k_1$. That means, the thermodynamic parameters we determined would not be related to k_1 alone, but also to k_2 and k_{-1}.

Two arguments stand however against that model: the spectral amplitude of the kinetics corresponded well to the amplitude measured at equilibrium, i.e., we did not observe any trace of a very fast first phase. Furthermore, the induced fit model may not describe adequately the binding of small ligands to hemoproteins. Instead, as indicated by the flash photolysis experiments of Frauenfelder, the rate-limiting step of CO binding at room temperature is the diffusion of CO through the protein and not the rearrangement of the enzyme-CO complex. The strictly linear relationship of k_{obs} as a function of the CO concentration which we always observed, confirms this hypothesis. For these reasons, it appears that the simpler one step model is sufficient to account for the kinetic data of CO binding to hemoproteins.

For most of the enzymes studied the binding rates agree with previously reported work. For cytochrome P-450 LM3c,rif and cytochrome P-450 7α, the CO binding kinetics were determined for the first time. In contrast to our stopped-flow results, the flash photolysis experiments on the CO complex of myoglobin by Ehrenstein et al. (1991) indicated a mechanism which comprised several steps: diffusion through the solvent, passage through a fluctuating protein, and binding from inside the heme pocket. However, the interpretation of the rebinding kinetic data is complicated by the fact that one does not know the position of the CO molecule after the flash. In stopped-flow experiments the situation is clear: all CO molecules are initially outside the protein. Nevertheless, as shown by the example of CO binding to P450 LM2, these two experimental approaches can lead to comparable results.

The effect of temperature

As shown in Fig. 5, the activation enthalpy, $\Delta H^{\ddagger}$, and the activation entropy, $\Delta S^{\ddagger}$, of the CO binding reaction are correlated by a compensation effect. From the slope of $\Delta H^{\ddagger} = f(\Delta S^{\ddagger})$ a compensation temperature of $T_c = 305$ K is calculated. Such compensation effects are observed frequently for enzyme kinetics, and their physical meaning is not clear. However, it appears from this figure, that the enthalpy/entropy compensation is the same for the various hemopro-

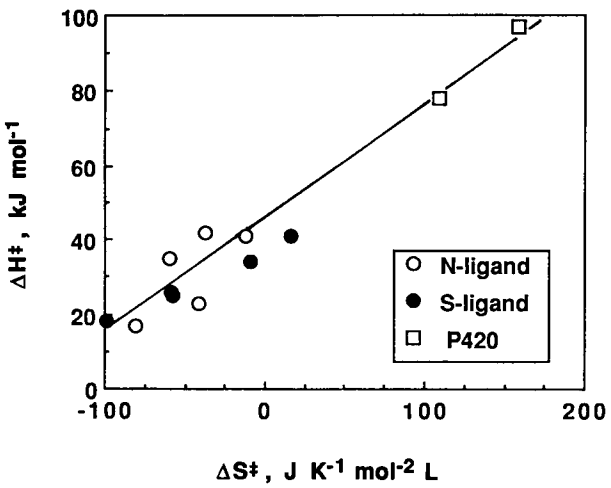

FIGURE 5 Correlation between the activation enthalpy $\Delta H^{\ddagger}$ and the activation entropy $\Delta S^{\ddagger}$ for the CO binding to hemoproteins. The N- and S-ligand classes, as well as cytochrome P-420 are distinguished by different symbols.

teins of our study, whatever the nature of their proximal axial ligand. A characterization of the transition state solely by $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ is therefore not very informative.

The effect of pressure

On the other hand, the effect of pressure on the binding rate constants clearly distinguishes between the sulfur and the nitrogen ligand hemoproteins (see Fig. 6). In the sulfur ligand class, the volume of the transition state is not significantly different from that of the resting enzyme. The invariance of $\Delta V^{\ddagger}$ as a function of the solvent polarity, the viscosity, and the salt concentration indicates further, that the interaction of the enzyme with the solvent is not changed in the transition state. The salt concentration, for instance, is known to affect the activation volume when exposure to solvent of charged residues is changed in the transition state (Low and Somero, 1975, 1975a). In the nitrogen ligand class, in contrast, the volume of the transition state is significantly smaller than that of the initial enzyme. It should be mentioned however, that proteins are relatively incompressible: for example, a volume decrease of 20 ml mol^{-1} (as observed typically for the N-ligand class) for a globular protein of 50 kDa corresponds to a volume change of only 0.04%. The same volume change is however too great to be explained by a modification of the rigid heme geometry, suggesting therefore that a protein conformational change or a modification of the protein solvation is involved. A similar distinction of these two classes had been made on the basis of the CO dissociation rate k_{off} ($=k_{-1}$) (Campbell et al., 1982) which is greater in the S-ligand class than in the N-ligand class.

In this study we have paid a particular attention to cytochrome P-450_{scc}. In a previous thermodynamic investigation, we had characterized the dynamics and the nature of conformational changes which occur as a result of changes in pH and temperature (Lange et al., 1992, 1992a). This study revealed that the latter parameters had no effect on the activation volume of the binding rate constant. It appears therefore that, in the sulfur ligand class, the transition state is structurally very close to the ground state. For the N-class, however, the activation volume depends on temperature, solvent, and viscosity (Balny and Travers, 1989), and hence on the protein conformation and/or its interaction with the solvent. Protein conformational changes are indeed often characterized by significant changes of the transition state volume (Butz et al., 1988; Unno et al., 1990).

Electronic effects of the axial S-ligand

It may appear surprising that the properties associated with the formation of a transition state of a simple reaction (the binding of CO to heme) suggest the involvement of protein conformational changes in the N-ligand hemoproteins, whereas practically no structural changes are observed with the S-ligand hemoproteins. The explanation may come from

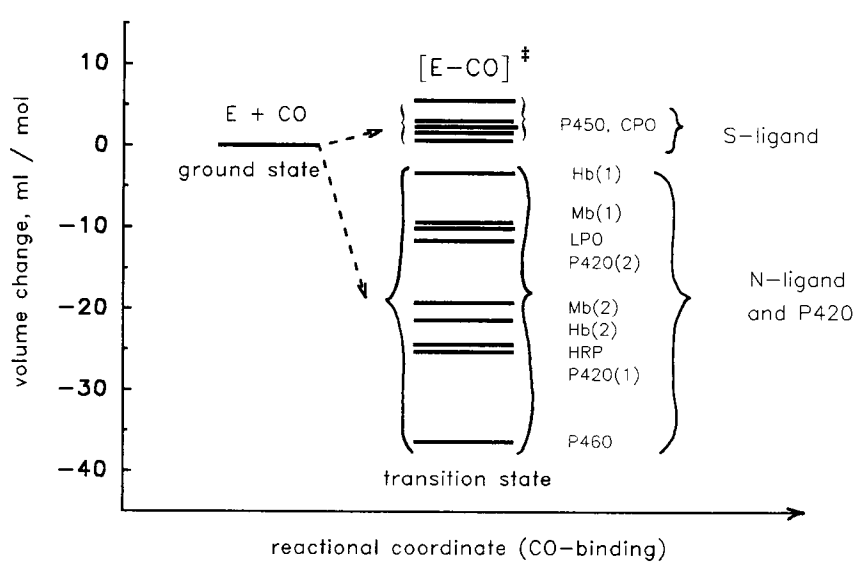

FIGURE 6 Variation of the transition state volume for various hemoproteins. Mb, myoglobin; Hb, hemoglobin; the numbers in parentheses refer to the kinetic phase related to. For other abbreviations see Tables 1 and 2.

the different electronic structure of S- and N-ligated hemes. The most striking difference between S-ligand hemoproteins and N-ligand hemoproteins is the strongly red-shifted Soret band of the ferrous CO complex (to 446–452 nm) in the former. Quantum chemical investigations by the Iterative Extended Hückel Theory (IEHT) suggest that electrons from high energy sulfur 3p orbitals participate in the aromatic porphyrin system, and that some electron density is transferred to the iron by sulfur $3p^+ \rightarrow$ porphyrin $e_g(\pi^*)$ charge transfer transition (Hanson et al., 1976; Rein et al., 1984). Using another theoretical approach, the π-INDO method, Jung et al. (1983) came to the conclusion that the negative charge from the mercaptide sulfur is donated partly to the CO ligand, resulting in a net charge of −0.223, whereas the CO ligand in hemoglobin has a net charge of only −0.155.

The significance of charge transfer is however not yet clear: recent investigations of the CO stretching modes by FTIR did not reveal the expected lowered vibration frequency (Jung et al., 1992). Furthermore, the stability as well as the absorption coefficients of charge transfer complexes of small organic molecules have been shown to depend on pressure (Ewald, 1968). But, as shown by our results, the CO binding to S-ligand hemoproteins is almost pressure insensitive. In addition, we did not observe any effect of pressure on the absorption spectrum of CO bound cytochrome P-450 (Fig. 7). Thus, whereas there is no doubt that the negatively charged sulfur ligand strongly affects the electronic configuration of the ferrous CO complex, the question is still open whether this effect is mediated by an electron charge transfer.

So, if changes in the conformation and in the interaction with the solvent are negligible for the S-ligand hemoproteins, it is probable that the CO binding transition state of this class of hemoproteins has a very particular electronic distribution which is favorable for the CO binding. Support for this hypothesis comes from our observation that when the sulfur-iron bond is broken or disturbed (as in the case of cytochrome P-420) (Jung et al., 1979), the enzyme activation is characteristic of a hemoprotein of the histidine class: for the three kinetic phases we observed negative $\Delta V^{\ddagger}$ values.

Approaches to study enzyme transition states

The difference in pressure dependence of the CO binding transition state between the S- and the N-ligand hemoprotein classes suggests that these two classes have adopted different strategies in the formation of an efficient transition state: the S-ligand class through an electronic effect-induced by a negative charge on the sulfur ligand- and the N-ligand class through protein conformational and/or solvation processes. Recent other approaches to the study of enzyme transition states focus on the pre-exponential factor A of the Arrhenius equation. Interpretations of kinetic results by the use of the Kramers equations (Kramers, 1940; Marden and Hui Bon Hoa, 1982) indicated that factor A depends on the viscosity of the solvent, and is thus related to the interaction of the enzyme with the solvent. Furthermore in their theoretical use of the stochastic theory, applied to enzyme reaction kinetics, Dunker et al. (1980) recommended the investigation of the prefactor when a multiplicity of protein conformation is suspected. Whereas these approaches are interesting for a given enzyme, our results indicate that a study of the preexponential factor alone is not informative in a comparative study of different enzymes. In fact, our measured values of the activation entropy $\Delta S^{\ddagger}$, which is related to A by

$$\Delta S^{\ddagger} = R\{\ln(Ah/k_B T) - 1\} \qquad (5)$$

where h and k_B are the Planck and the Boltzman constants, respectively, show a great variability between the various enzymes, but with no apparent correlation to structural features. In contrast, a kinetic investigation combining pressure and temperature effects appears to correlate with structural features and thus, perhaps, be more likely to lead to a more general understanding of the nature of enzyme reactional transition states. Progress towards this goal should result

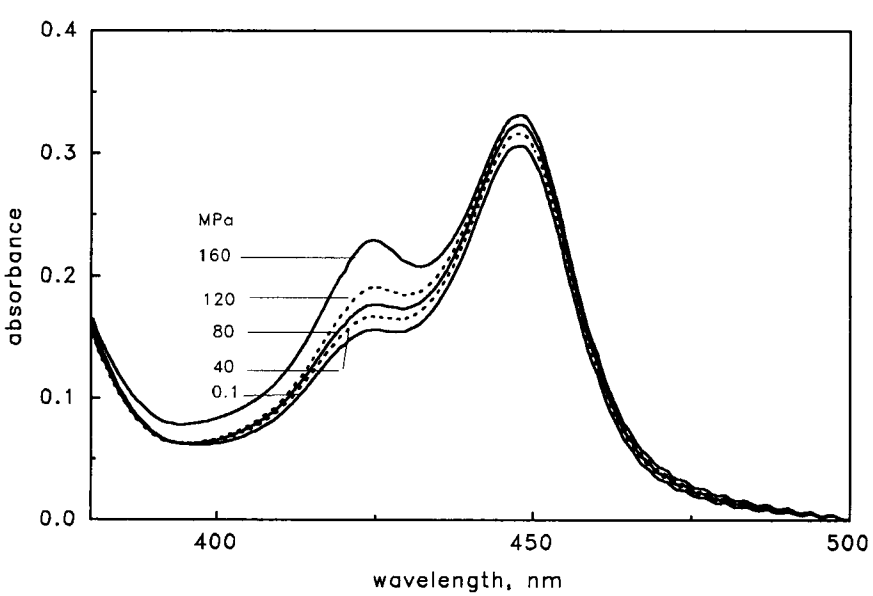

FIGURE 7 Pressure effect on the [cytochrome P-450$_{scc}$-CO] complex. Cytochrome P-450$_{scc}$, 3 μM, was reduced by sodium dithionithe (grains), and the CO complex was formed by bubbling CO through the solution for some seconds; the buffer was potassium phosphate, 50 mM, pH 7.4 at 25°C. The spectra were recorded under various pressures, as indicated in the figure, in a home built high pressure cell with a Cary 3 spectrophotometer.

from more studies of the properties of reactional transition states of many enzymes by the use of high pressure fast kinetic methods. The necessary technology has been available for several years (Heremans, 1992).

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