Antagonistic Effects of Hydrostatic Pressure and Osmotic Pressure on Cytochrome P-450$_{cam}$ Spin Transition

Carmelo Di Primo, Eric Deprez, Gaston Hui Bon Hoa, and Pierre Douzou
INSERM-INRA U310, Institut de Biologie Physico-Chimique, 75005 Paris, France

ABSTRACT The combined effects of hydrostatic pressure and osmotic pressure, generated by polyols, on the spin equilibrium of fenchone-bound cytochrome P-450$_{cam}$ were investigated. Hydrostatic pressure induces a high spin to low spin transition, whereas polyols induce the reversed reaction. Of the four solutes used, glycerol, glucose, stachyose, and sucrose, only the last two would act on the spin transition by osmotic stress. The spin volume changes measured by both techniques are different, 29 and −350 ml/mol for hydrostatic pressure and osmotic pressure, respectively. It suggests that even if the two are perturbing water molecules, different properties are probed. From the volume change induced by osmotic stress, 19 water molecules are deduced that would be implicated in the spin transition of the fenchone-bound protein. This result suggests that water molecules other than the well defined ones located in the active site play a key role in modulating the spin equilibrium of cytochrome P-450$_{cam}$.

INTRODUCTION

Cytochrome P-450$_{cam}$ is a soluble hemoprotein from *Pseudomonas putida* that catalyzes the hydroxylation of its physiological substrate, camphor, with high regiospecificity (Gelb et al., 1982). The three-dimensional x-ray structure has been determined in both the substrate-free (Poulos et al., 1986) and substrate-bound (Poulos et al., 1985, 1987) forms as well as when bound with other ligands (Poulos and Howard, 1987; Raag and Poulos, 1989). In the camphor-free form, the heme pocket is occupied by a network of six water molecules, one of which is coordinated to the heme iron as its sixth ligand. When camphor binds, the water molecules are removed from the active site, leading to a pentacoordinated heme iron. This reaction, known as the spin transition between a substrate-free low spin form and a substrate-bound high spin form (Tsai et al., 1970; Sligar and Gunsalus, 1976), constitutes the first step of the hydroxylation cycle of camphor (Unger et al., 1986; Gunsalus et al., 1987).

The spin equilibrium displayed by cytochrome P-450$_{cam}$ is extremely sensitive to physical chemical agents (Hui Bon Hoa and Marden, 1982; Marden and Hui Bon Hoa, 1982; Fisher et al., 1985; Fisher and Sligar, 1987). Among them, hydrostatic pressure is probably the most versatile perturbation used to investigate the binding step of the substrate and the associated spin transition. When hydrostatic pressure is applied to the camphor-bound form of the protein, the spin equilibrium is shifted from high spin to low spin (Hui Bon Hoa et al., 1989; Di Primo et al., 1990). Up to 1500 bar, this displacement is reversible as soon as pressure is released. In this range, the protein is stable and stays native. Beyond 1500 bar, cytochrome P-450$_{cam}$ converts into an inactive but soluble form known as cytochrome P-420. In the 1–1500 bar range of pressure where the protein is native, the induced spin transition can be quantitated by measuring the variation of the spin equilibrium constant, K_{spin}, as a function of hydrostatic pressure. This will give the volume change of the spin transition, ΔV_{spin}, according to the equation $\partial \ln K_{spin}/\partial P = -\Delta V_{spin}/RT$.

The effect of hydrostatic pressure on biomolecules is well documented. This technique, used to investigate protein stability and ligand-protein interactions (Heremans, 1982; Weber and Drickamer, 1983), is also particularly suitable for the analysis of protein-protein interactions. When submitted to an increase in hydrostatic pressure, protein-protein complexes generally dissociate because of the solvation of their interacting surfaces (Kornblatt et al., 1988; Rodgers and Sligar, 1991). Results obtained with monomeric proteins show that structural perturbations induced by hydrostatic pressure are also accompanied with changes in solvation (Kundrot and Richards, 1988). In cytochrome P-450$_{cam}$, these hydration changes occur in an active site that is entirely buried in the protein with no direct access to the solvent (Fisher et al., 1985; Hui Bon Hoa et al., 1992; Di Primo et al., 1992a).

In the 1980s, a new approach was developed to investigate the role of water molecules in biological reactions (Parsegian et al., 1986; Rand and Parsegian, 1989). This approach, known as the osmotic stress strategy, uses osmolytes, mainly polyols, to decrease the activity of water. The generated osmotic stress will affect any osmotically active water molecules that are implicated in the activity of the protein. By measuring characteristic parameters of the reaction such as equilibrium constants and/or kinetic constants as a function of osmotic pressure, it is possible to determine the number of water molecules associated to the protein that play a key

Received for publication 2 November 1994 and in final form 6 February 1995.

Address reprint requests to Carmel Di Primo, INSERM-INRA U310, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France. Tel: 33-1-43-25-2609; Fax: 33-1-43-29-8088; E-mail: diprimo@ibpc.fr.

Abbreviations used: LS, low spin; HS, high spin; $\Delta V_{spin\ osm}$, spin volume change induced by osmotic pressure; $\Delta V_{spin\ hyd}$, spin volume change induced by hydrostatic pressure; P_o, osmotic pressure; P_h, hydrostatic pressure.

0006-3495/95/05/2056/06 $2.00

role in the reaction. Numerous examples have demonstrated the validity of this methodology as in the cytochrome *c* oxidase reaction (Kornblatt and Hui Bon Hoa, 1990), the binding of oxygen to hemoglobin (Colombo et al., 1992), cytochrome P-450$_{cam}$ (Di Primo et al., 1992b), the interaction of DNA with the restriction endonuclease *Eco*RI (Robinson and Sligar, 1993), protein-protein complexes (Kornblatt et al., 1993a), and the binding of glucose to hexokinase (Rand et al., 1993).

If hydrostatic pressure, which shifts equilibria toward the hydrated forms, and osmotic pressure, which has an opposite effect, have been combined recently to analyze the role of water in the dissociation of enolase, a dimeric enzyme (Kornblatt et al., 1993b) and in the specificity of *Eco*RI-DNA interactions (Robinson and Sligar, 1994), no work has been dedicated to compare in a two-state equilibrium process the volume change for the reaction induced by hydrostatic pressure with the volume change for the reversed reaction induced by osmotic pressure. Based on the results mentioned above, which show that hydrostatic pressure shifts the spin equilibrium of cytochrome P-450$_{cam}$ toward a more hydrated active site (low spin form), and on preliminary observations that indicate that polyols, on the contrary, influence the spin equilibrium toward a less hydrated active site (high spin form), experiments that combine both perturbations were achieved first to determine whether the effect of polyols on the spin transition are related to an osmotic stress and then to compare the volume change that results from each perturbation.

MATERIALS AND METHODS

Cytochrome P-450$_{cam}$ was generated and purified as described previously (Gunsalus and Wagner, 1978). The substrate-free protein was obtained by passage of one sample of pure concentrated protein through a Sephadex G-25 column previously equilibrated at 4°C with the buffer used for the experiments, 100 mM Tris-HCl (Sigma Chemical Co., St Louis, MO), pH 7, at 20°C.

To observe the effect of hydrostatic pressure and osmotic pressure, it was crucial to find experimental conditions where the spin equilibrium is sensitive to both parameters. The substrate-free form of cytochrome P-450$_{cam}$, which has a fully hydrated active site (100% low spin), converts into cytochrome P-420 as soon as pressure is applied (Hui Bon Hoa et al., 1989; Di Primo et al., 1990). Camphor-bound cytochrome P-450$_{cam}$ has a totally dehydrated active site (100% high spin) (Poulos et al., 1986; Di Primo et al., 1990). The transition toward a more hydrated form, induced by hydrostatic pressure, can be followed but, obviously, a transition toward a less hydrated heme pocket, induced by cosolvents, could not be followed. For the experiments, a structural analog of camphor, fenchone (Janssen Biochimica, Berse, Belgium), was used. At saturating concentration of this substrate, 1.3 mM, in the presence of 240 mM potassium, cytochrome P-450$_{cam}$ displays a spin equilibrium between 25% low spin and 75% high spin, and the protein stays native up to 1500 bar in aqueous medium (Di Primo et al., 1992a).

The high spin to low spin transition induced by hydrostatic pressure was measured by using a Cary 219 spectrophotometer interfaced with a high-pressure bomb as described previously (Hui Bon Hoa et al., 1982). The thermal regulation of the bomb, at 20°C, was provided by a commercial thermostated circulating bath (Huber HS 40), allowing a temperature control of ±0.5°C. The hydrostatic pressure was applied by 100- to 200-bar increments. The data were collected and analyzed on a PC. Software was used to calculate the content of high spin and low spin as a function of hydrostatic pressure (Davydov et al., 1992). The spin volume change of the transition, $\Delta V_{spin\ hyd}$, is determined by fitting the variation of ln K_{spin} as a function of hydrostatic pressure to the equation:

$$\frac{\partial \ln K_{spin}}{\partial P_h} = -\frac{\Delta V_{spin\ hyd}}{RT} \quad (1)$$

where $K_{spin} = HS/LS$ is the spin equilibrium constant that describes the two-state equilibrium $LS \leftrightarrow HS$ and P_h is the hydrostatic pressure.

Four polyols were used to generate the osmotic pressure: glycerol (FW 92.09, Carlo Erba), stachyose (FW 738.64, Janssen), glucose, and sucrose (FW 180.16, 342.30, respectively, Sigma). The values of osmotic pressure were measured with a Roebling osmometer or were calculated from the *Handbook of Chemistry and Physics*, 52nd Edition, 1971–1972 data as described previously (Fullerton et al., 1992). The cosolvents were added slowly to the fenchone-bound protein samples at 4°C to prevent inactivation of cytochrome P-450$_{cam}$. At each osmotic pressure, a hydrostatic pressure experiment was performed. This permitted us to determine the spin equilibrium constant, $K_{spin} = HS/LS$, as a function of osmotic pressure, P_o, and the volume change of the spin transition induced by osmotic pressure, at constant hydrostatic pressure, according to the equation:

$$\frac{\partial \ln K_{spin}}{\partial P_o} = -\frac{\Delta V_{spin\ osm}}{RT} \quad (2)$$

RESULTS

The high spin to low spin transition induced by hydrostatic pressure on fenchone-bound cytochrome P-450$_{cam}$ in aqueous medium is presented in Fig. 1. At one bar the spectrum is characteristic of a mixture of low spin and high spin forms that absorb at 417 and 392 nm, respectively. The spectrum at atmospheric pressure reflects a mixture of 75% high spin

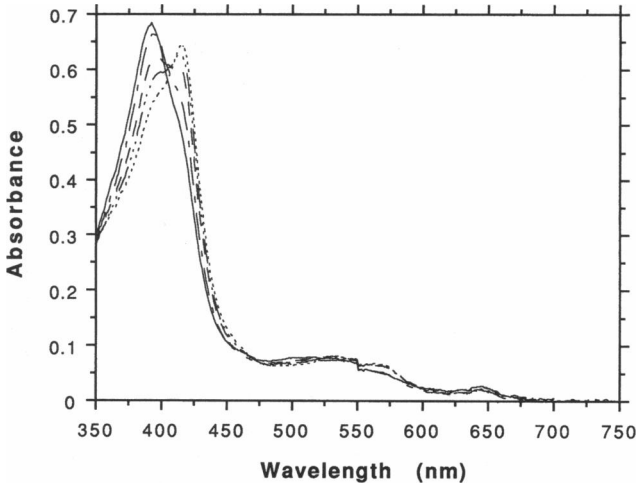

FIGURE 1 Absorption spectra of fenchone-bound cytochrome P-450$_{cam}$ as a function of hydrostatic pressure. Experimental conditions are: cytochrome P-450$_{cam}$ 8 μM, fenchone 1.3 mM, KCl 240 mM in Tris-HCl 100 mM pH 7 at 20°C. At 1 bar (——) the spectrum presents an absorption maximum at 392 nm and shoulder at 417 nm, which are characteristic of the high spin and low spin forms, respectively, with a clear isosbestic point at 404 nm. This spectrum reflects a mixture of high spin (75%) and low spin (25%). When hydrostatic pressure is applied, the spin equilibrium is shifted toward low spin as seen in the decrease in the absorption at 392 nm and the increase at 417 nm. (— - —) 250 bar, (— —) 500 bar, (- ··· -) 750 bar, (- - - -) 1250 bar.

and 25% low spin forms. When the hydrostatic pressure is increased, cytochrome P-450$_{cam}$ undergoes a spin transition from high spin (392 nm) to low spin (417 nm) with a clear isosbestic point at 404 nm. In aqueous medium, between 1 and 1500 bar the transition is reversible as soon as the pressure is released.

The combined effects of hydrostatic pressure and concentration of sucrose on fenchone-bound cytochrome P-450$_{cam}$ is shown in Fig. 2. Similar curves were obtained with glycerol, glucose, and stachyose (data not shown). As hydrostatic pressure is increased, the spin equilibrium is shifted toward the low spin forms, ln K_{spin} decreases, whereas increasing concentrations of sucrose induce a low spin to high spin transition as seen in the increase in ln K_{spin} at 1 bar as well as at any other hydrostatic pressure value. The volume change of the transition, $\Delta V_{spin\ hyd}$, induced by hydrostatic pressure, at each concentration of polyol, was deduced by fitting these curves to Eq. 1 in the significant pressure-sensitive region of the spin transition, beyond 500 bar. For each concentration of polyol, the corresponding osmotic pressure was determined as described in Materials and Methods. $\Delta V_{spin\ hyd}$ was then plotted as a function of osmotic pressure as presented in Fig. 3. Within the experimental error, the increase in osmotic pressure generated by glycerol, glucose, stachyose, or sucrose does not significantly influence $\Delta V_{spin\ hyd}$, the value of which is equal to 29 ± 3 ml/mol.

The effect of osmotic pressure on the spin equilibrium constant, K_{spin}, at constant hydrostatic pressure is presented in Fig. 4 for all of the polyols used, glycerol, glucose, stachyose, and sucrose. The combined effect of osmotic pressure and hydrostatic pressure is seen again in these plots. The increase in ln K_{spin} as a function of osmotic pressure reflects the low spin to high spin transition induced by the polyols. At constant osmotic pressure, the decrease in ln K_{spin} as a function of hydrostatic pressure reflects the hydrostatic-induced high spin to low spin transition. Between 1 and 30 bar, glycerol (Fig. 4 *a*) does not shift the spin equilibrium. Only the points from 30 bar were taken into account to determine the volume change of the spin transition induced by osmotic pressure, $\Delta V_{spin\ osm}$. This parameter was calculated from the slopes of these lines according to Eq. 2. For glucose (Fig. 4 *b*), and for similar reasons, only the points from 24 bar were considered. Stachyose (Fig. 4 *c*) and sucrose (Fig. 4 *d*) both induce a low spin to high spin transition in the osmotic pressure range, where no effect was observed with glycerol and glucose. $\Delta V_{spin\ osm}$ deduced from these slopes was then plotted as a function of hydrostatic pressure as presented in Fig. 5. $\Delta V_{spin\ osm}$ increases, in absolute value, with hydrostatic pressure. At constant hydrostatic pressure, $\Delta V_{spin\ osm}$ increases in absolute value going from a small polyol to a larger one. Interestingly, sucrose (FW 342.30) and stachyose (FW 738.64) behave similarly. This point will be discussed in the next section.

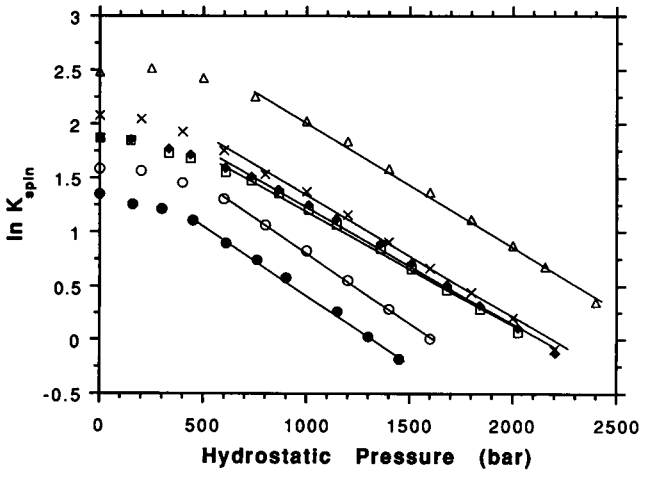

FIGURE 2 ln K_{spin} as a function of hydrostatic pressure in increasing concentration of sucrose. (●) sucrose 0%, (○) sucrose 23%, (◆) sucrose 30.5%, (□) sucrose 39%, (×) sucrose 41%, (Δ) and sucrose 48%. The concentrations of sucrose are given by weight. All of the experiments were done in 100 mM Tris-HCl, pH 7, at 20°C, 240 mM KCl, and 1.3 mM fenchone.

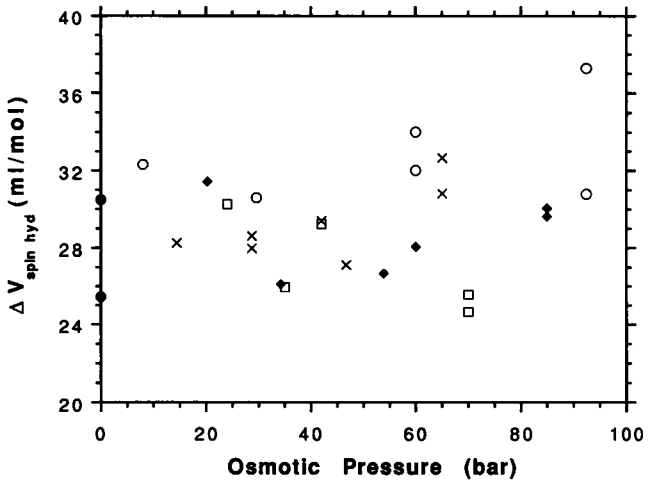

FIGURE 3 $\Delta V_{spin\ hyd}$ as a function of osmotic pressure. (□) glucose, (◆) sucrose, (×) stachyose, (●) 100 mM Tris-HCl, (○) and glycerol. $\Delta V_{spin\ hyd}$ was deduced by fitting to Eq. 1 curves similar to those presented in Fig. 2 in the significant pressure-sensitive region of the transition.

DISCUSSION

The effect of hydrostatic pressure on the spin transition displayed by cytochrome P-450$_{cam}$ is well established (Hui Bon Hoa and Marden, 1982; Di Primo et al., 1990). The transition from high spin to low spin as a function of this perturbation results in a hydration change of the active site of the protein (Fisher et al., 1985; Hui Bon Hoa et al., 1992; Di Primo et al., 1992a). On the contrary, polyols induce a transition from low spin to high spin. Because these two perturbations have opposite effects, an obvious goal was to quantitate and then to compare their effects on the simplest reaction of the catalytic cycle of cytochrome P-450$_{cam}$, the spin transition. The initial question to address was whether the effect of polyols on this transition was related to an induced osmotic stress.

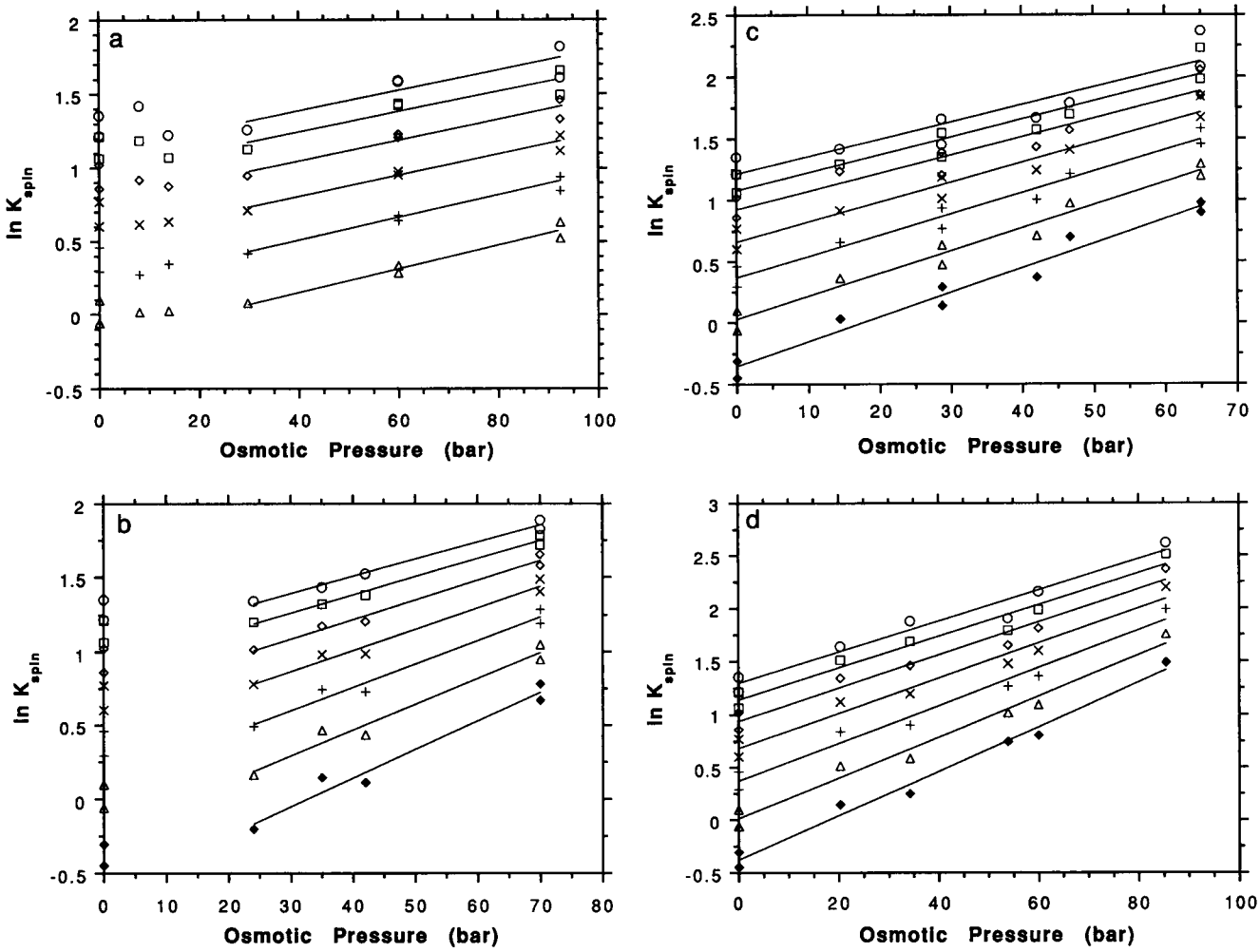

FIGURE 4 ln K_{spin} as a function of osmotic pressure at increasing hydrostatic pressure. (*a*) Osmotic pressure generated by glycerol. (*b*) Osmotic pressure generated by glucose. (*c*) Osmotic pressure generated by stachyose. (*d*) Osmotic pressure generated by sucrose. Hydrostatic pressure values: (○) 1 bar, (□) 250 bar, (◇) 500 bar, (×) 750 bar, (+) 1000 bar, (Δ) 1250 bar, and (◆) 1500 bar.

As seen in Fig. 4, two of the four polyols used to generate the osmotic stress, stachyose (Fig. 4 *c*) and sucrose (Fig. 4 *d*), display a good linearity between ln K_{spin} and osmotic pressure. For glycerol (Fig. 4 *a*) and glucose (Fig. 4 *b*), the linearity does not extend from 0 bar to the highest osmotic stresses. These results suggest that only stachyose and sucrose are good osmolytes. This conclusion is supported by the spin volume change induced by osmotic pressure, $\Delta V_{spin\ osm}$, found with these two polyols, the values of which are presented in Fig. 5. For sucrose and stachyose, $\Delta V_{spin\ osm}$ does not depend on the size of the solute. A possible explanation for why glycerol and glucose are not good osmolytes is that these molecules access small spaces around the protein. It was proposed that depending on the size of the osmolyte, different size aqueous compartments could be detected around the protein (Rand et al., 1993). Because with stachyose and sucrose the linearity of ln K_{spin} versus osmotic pressure extends from 0 to the highest stresses and the corresponding spin volume changes are equal, these larger probes are probably totally excluded from the spaces seen by glycerol and glucose.

To explain the absence of an osmotic pressure effect on $\Delta V_{spin\ hyd}$ (see Fig. 3), we should first consider that the applied mechanical hydrostatic pressure is not influenced by any means by medium conditions such as changes in osmotic pressure. Then, as was shown previously (Hui Bon Hoa et al., 1992), only the initial percentage of high spin would determine the value of the spin volume change obtained with hydrostatic pressure, $\Delta V_{spin\ hyd}$. This parameter increases, in absolute value, with increasing amounts of high spin forms. Osmotic pressure influences the spin equilibrium. At the highest stress, this equilibrium is shifted toward high spin by 15%. However, within the experimental sensitivity of our hydrostatic pressure apparatus, this is too small a shift to detect a change in $\Delta V_{spin\ hyd}$.

On the contrary, thermodynamics predict that osmotic pressure depends on hydrostatic pressure. Unfortunately, we could only measure the osmotic pressures at atmospheric pressure. The exact reason of the variations of $\Delta V_{spin\ osm}$ as a function of hydrostatic pressure (see Fig. 5) is not clear. Are the osmotic pressure values at higher hydrostatic pressure simply underestimated? This would be the case if the os-

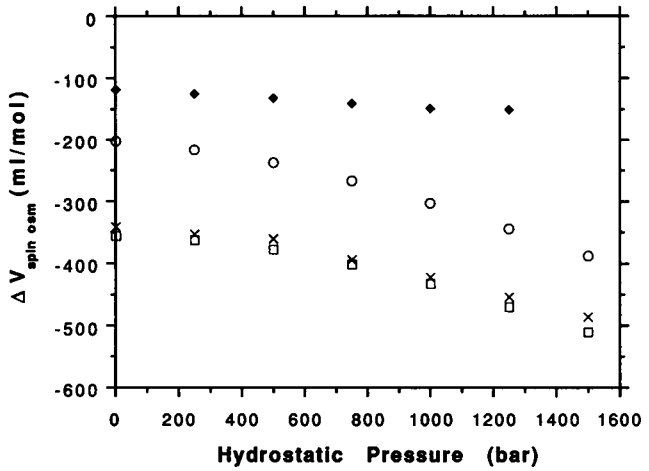

FIGURE 5 $\Delta V_{spin\ osm}$ as a function of hydrostatic pressure. (♦) Glycerol, (○) glucose, (×) stachyose, and (□) sucrose. At each hydrostatic pressure, $\Delta V_{spin\ osm}$ was calculated according to Eq. 2 from the lines of the plots presented in Fig. 4.

molality of the solution increases at higher hydrostatic pressure, because of the contraction of the sample. Does the solute exclusion depend on hydrostatic pressure?

Recently, it was found that 400 bar of hydrostatic pressure was required to counteract the effect of 100 bar of osmotic pressure on the specificity of *Eco*RI-DNA interactions (Robinson and Sligar, 1994). In this work also, more hydrostatic pressure is required to counteract the effect of osmotic pressure. 900 bar of hydrostatic pressure in aqueous medium shifts the spin equilibrium of cytochrome P-450$_{cam}$ by 15% toward low spin, whereas only 100 bar of osmotic pressure, at 1 bar of hydrostatic pressure, shifts the spin equilibrium of the same percentage toward high spin. This antagonistic effect and the amount of each pressure required to shift the spin equilibrium of an equivalent percentage are both reflected in the volume change. $\Delta V_{spin\ osm}$ and $\Delta V_{spin\ hyd}$ have opposite signs. $\Delta V_{spin\ hyd}$ is smaller, in absolute value, than $\Delta V_{spin\ osm}$ at atmospheric pressure: 29 and 350 ml/mol at atmospheric pressure, respectively.

Clearly, each perturbation is not probing the same process. The volume change measured with hydrostatic pressure is a complex parameter. However, it is generally admitted that this parameter reflects the sum of at least two contributions (Low and Somero, 1975): a conformational one that corresponds to the physical volume change resulting from the contraction of the system submitted to pressure, and a hydration one as when groups are exposed to the solvent. Electrostriction phenomena correspond to the exposure of charged groups. The measured volume change results then from density changes of water molecules within the system (Stranks, 1974). In cytochrome P-450$_{cam}$, we have shown that part of $\Delta V_{spin\ hyd}$ is correlated to the initial hydration degree of the heme pocket (Hui Bon Hoa et al., 1992). This indicates that density changes of water molecules between the bulk solvent and the heme pocket probably contribute to the measured $\Delta V_{spin\ hyd}$. We still cannot determine the part due to this process and the one attributed to the conformational change that the protein undergoes, as suggested by fluorescence studies of cytochrome P-450$_{cam}$ under hydrostatic pressure (Hui Bon Hoa et al., 1989).

The volume change measured with osmotic pressure would rather reflect the protein-bound water molecules displaced when the activity of water is decreased. The number of these osmotically active water molecules can be estimated from $\Delta V_{spin\ osm}$ taking the molar partial volume of water as 18 ml/mol (Colombo et al., 1992; Kornblatt and Hui Bon Hoa, 1990, Rand et al., 1993). 19 water molecules, deduced from $\Delta V_{spin\ osm}$ at atmospheric pressure, for stachyose and sucrose would be displaced during the low spin to high spin transition of the fenchone-bound cytochrome P-450$_{cam}$. In the substrate-free protein, four water-binding sites are present in the active site. Two were experimentally observed by x-ray crystallography (Poulos et al., 1986, 1987) and predicted by the molecular dynamics method (Wade, 1990). They correspond to regions A (one water molecule) and B (six water molecules). Two others were only predicted, regions C and D (one water molecule each) (Wade, 1990). This would account for a total of nine water molecules. Only the six water molecules from region B are displaced during the spin transition upon binding of camphor (Poulos et al., 1987). The fenchone-bound x-ray structure is not available, but considering that this substrate analog does not induce a complete spin transition to 100% high spin as camphor does, the water occupancy of the active site is expected to be between nine (substrate-free active site) and three (camphor-bound active site) water molecules. However, this is less than the 19 water molecules found by the osmotic stress strategy. Then part of this number, or perhaps all, would concern other structural water molecules than those located in the active site, whose osmotically perturbed interactions with the protein are able to influence the spin equilibrium. This would be consistent with a recent work that revealed that the presence of water in the active site, especially as the sixth ligand of the heme iron, is not sufficient to explain the low spin resting state of cytochrome P-450$_{cam}$ and that the protein itself plays a crucial role in modulating the spin equilibrium (Harris and Loew, 1993).

In conclusion, we have shown that osmotic pressure and hydrostatic pressure have antagonistic effects on the spin equilibrium of cytochrome P-450$_{cam}$. Two of the four polyols used, stachyose and sucrose, would act on the spin transition by osmotic stress. The volume changes measured by osmotic pressure and hydrostatic pressure are different. This suggests that two different water properties are probed. Hydrostatic pressure probes differences in density, whereas osmotic pressure probes a difference in the number of water molecules associated with two protein conformations. 19 water molecules would be involved in the spin transition, suggesting that water molecules other than the well defined ones located in the active site play a key role in modulating the first step of the hydroxylation cycle of camphor by cytochrome P-450$_{cam}$.

We thank Drs. Judith Kornblatt, Jack Kornblatt, Pascale Debey, Peter Rand, and Adrian Parsegian for helpful discussions and a critical reading of the manuscript. We gratefully thank the anonymous reviewer and Dr. Bernard Alpert for their crucial comments. We thank Laurence Mathivet and Dr. Alain Zachowski for the use of the ROEBLING osmometer. We also thank Dr. Sligar, who provided us all of the facilities of his laboratory at the University of Illinois Urbana-Champaign to generate the protein.
This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Institut National de la Recherche Agronomique, and grant from the European Community BI02-CT94-2060. E. Deprez is supported by the Ministère de la Recherche et de l'Espace.

REFERENCES

Colombo, M. F., D. C. Rau, and V. A. Parsegian. 1992. Protein solvation in allosteric regulation: a water effect on haemoglobin. *Science.* 256: 655–659.

Davydov, D. R., T. V. Knyushko, and G. Hui Bon Hoa. 1992. High pressure induced inactivation of ferrous cytochrome P-450 LM2 (IIB4) CO complex: evidence for the presence of two conformers in the oligomer. *Biochem. Biophys. Res. Commun.* 188:216–221.

Di Primo, C., G. Hui Bon Hoa, P. Douzou, P., and S. G. Sligar. 1990. Effect of tyrosine 96 hydrogen bond on the inactivation of cytochrome P-450$_{cam}$ induced by hydrostatic pressure. *Eur. J. Biochem.* 193:383–386.

Di Primo, C., G. Hui Bon Hoa, P. Douzou, and S. G. Sligar. 1992a. Heme-pocket-hydration change during the inactivation of cytochrome P-450$_{camphor}$ by hydrostatic pressure. *Eur. J. Biochem.* 209:583–588.

Di Primo, C., S. G. Sligar, G. Hui Bon Hoa, and P. Douzou. 1992b. A critical role of protein-bound water in the catalytic cycle of cytochrome P-450 camphor. *FEBS Lett.* 312:252–254.

Fisher, M. T., and S. G. Sligar. 1987. Temperature jump relaxation kinetics of the P-450cam spin equilibrium. *Biochemistry.* 26:4797–4803.

Fisher, M. T., S. F. Scarlata, and S. G. Sligar. 1985. High-pressure investigations of cytochrome P-450 spin and substrate binding equilibria. *Arch. Biochem. Biophys.* 240:456–463.

Fullerton, G. D., R. J. Zimmerman, C. Cantu, and I. L. Cameron. 1992. New expressions to describe solution non ideal osmotic pressure, freezing point depression, and vapour pressure. *Biochem. Cell. Biol.* 70:1325–1331.

Gelb, M. H., D. C. Heimbrook, P. Malkonen, and S. G. Sligar. 1982. Stereochemistry and deuterium isotope effects in camphor hydroxylation by the cytochrome P450cam monooxygenase system. *Biochemistry.* 21:370–377.

Gunsalus, I. C., and G. C. Wagner. 1978. Bacterial P-450cam methylene monooxygenase components: cytochrome m, putidaredoxin, and putidaredoxin reductase. *Methods Enzymol.* 52:166–188.

Gunsalus, I. C., H. Koga, and S. G. Sligar. 1987. Four Decades of P-450, Proceedings of the Yamada Conference. R. Sato, editor. Yamada Foundation, Tokyo, Japan. 142–149.

Harris, D., and G. Loew. 1993. Determinants of the spin state of the resting state of cytochrome P450$_{cam}$. *J. Am. Chem. Soc.* 115:8775–8779.

Heremans, K. 1982. High pressure effects on proteins and other biomolecules. *Annu. Rev. Biophys. Bioengerg.* 11:1–21.

Hui Bon Hoa, G., and M. C. Marden. 1982. The pressure dependence of the spin equilibrium in camphor-bound ferric cytochrome P-450. *Eur. J. Biochem.* 124:311–315.

Hui Bon Hoa, G., C. Di Primo, and P. Douzou. 1992. The role of electrostriction in the control of reaction volumes of biochemical processes. In Proceedings of the First European Seminar on High Pressure and Biotechnology, La Grande Motte, France. 139.

Hui Bon Hoa, G., C. Di Primo, I. Dondaine, S. G. Sligar, I. C. Gunsalus, and P. Douzou. 1989. Conformational changes of cytochrome P-450$_{cam}$ and P-450$_{lin}$ induced by hydrostatic pressure. *Biochemistry.* 28:651–656.

Hui Bon Hoa, G., P. Douzou, N. Dahan, and C. Balny. 1982. High-pressure spectrometry at subzero temperature. *Anal. Biochem.* 120:125–135.

Kornblatt, J. A., and G. Hui Bon Hoa. 1990. A non-traditional role for water in the cytochrome c oxidase reaction. *Biochemistry.* 29:9370–9376.

Kornblatt, J. A., G. Hui Bon Hoa, L. Eltis, and A. G. Mauk. 1988. The effects of pressure on porphyrin-c-cytochrome b_5 complex formation. *J. Am. Chem. Soc.* 110:5909–5911.

Kornblatt, J. A., M. J. Kornblatt, G. Hui Bon Hoa, and A. G. Mauk. 1993a. Responses of two protein-protein complexes to solvent stress: does water play a role at the interface? *Biophys. J.* 65:1059–1065.

Kornblatt, M. J., J. A. Kornblatt, and G. Hui Bon Hoa. 1993b. The role of water in the dissociation of enolase, a dimeric enzyme. *Arch. Biochem. Biophys.* 306:495–500.

Kundrot, C. E., and F. M. Richards. 1988. The effect of hydrostatic pressure on the solvent in crystals of hen egg-white lysozyme. *J. Mol. Biol.* 200: 401–410.

Low, P. S., and G. N. Somero. 1975. Activation volumes in enzymatic catalysis: their sources and modification by low-molecular-weight solutes. *Proc. Natl. Acad. Sci. USA.* 72:3014–3018.

Marden, M. C., and G. Hui Bon Hoa. 1982. Dynamics of the spin transition in camphor-bound ferric cytochrome P-450 versus temperature, pressure and viscosity. *Eur. J. Biochem.* 129:111–117.

Parsegian, V. A., R. P. Rand, N. L. Fuller, D. C. Rau. 1986. Osmotic stress for the direct measurement of intermolecular forces. *Methods Enzymol.* 127:400–416.

Poulos, T. L., and A. J. Howard. 1987. Crystal structures of metyrapone- and phenylimidazole-inhibited complexes of cytochrome P-450$_{cam}$. *Biochemistry.* 26:8165–8174.

Poulos, T. L., B. C. Finzel, and A. J. Howard. 1986. Crystal structure of substrate-free *Pseudomonas putida* cytochrome P-450. *Biochemistry.* 25: 5314–5322.

Poulos, T. L., B. C. Finzel, and A. J. Howard. 1987. High-resolution crystal structure of cytochrome P-450$_{cam}$. *J. Mol. Biol.* 195:687–700.

Poulos, T. L., B. C. Finzel, I. C. Gunsalus, G. C. Wagner, and J. Krant. 1985. The 2.6-Å crystal structure of *Pseudomonas putida* cytochrome P-450. *J. Biol. Chem.* 260:16122–16130.

Raag, R., and T. L. Poulos. 1989. Crystal structure of the carbon monoxide-substrate-cytochrome P-450$_{cam}$ ternary complex. *Biochemistry.* 28:7586–7592.

Raag, R., and T. L. Poulos. 1989. The structural basis for substrate-induced changes in redox potential and spin equilibrium in cytochrome P-450$_{cam}$. *Biochemistry.* 28:917–922.

Rand, R. P, N. L. Fuller, P. Butko, G. Francis, and P. Nicholls. 1993. Measured change in protein solvation with substrate binding and turnover. *Biochemistry.* 32:5925–5929.

Rand, R. P., and V. A. Parsegian. 1989. Hydration forces between phospholipid bilayers. *Biochem. Biophys. Acta.* 988:351–376.

Robinson, C. R., and S. G. Sligar. 1993. Molecular recognition mediated by bound water: a mechanism for star activity of the restriction endonuclease *Eco*RI. *J. Mol. Biol.* 234:302–306.

Robinson, C. R., and S. G. Sligar. 1994. Hydrostatic pressure reverses osmotic pressure effects on the specificity of *Eco*RI-DNA interactions. *Biochemistry.* 33:3787–3793.

Rodgers, K. K., and S. G. Sligar. 1991. Mapping electrostatic interactions in macromolecular associations. *J. Mol. Biol.* 221:1453–1460.

Sligar, S. G., and I. C. Gunsalus. 1976. Proton coupling in the cytochrome P-450 spin and redox equilibria. *Proc. Natl. Acad. Sci. USA.* 84:1078–1082.

Stranks, D. R. 1974. The elucidation of inorganic reaction mechanisms by high pressure studies. *Pure Appl. Chem.* 38:303–323.

Tsai, R., C. Yu, I. C. Gunsalus, J. Peisach, W. Blumburg, W. H. Orme-Johnson, and H. Beinert. 1970. Spin-state changes in cytochrome P-450$_{cam}$ on binding of specific substrates. *Proc. Natl. Acad. Sci. USA.* 66:1157–1163.

Unger, B., I. C. Gunsalus, and S. G. Sligar. 1986. The Bacteria, Vol. X. J. Sokatch, editor. Academic Press, New York. 557–587.

Wade, R. C. 1990. Solvation of the active site of cytochrome P-450-cam. *J. Computer-Aided Mol. Design.* 4:199–204.

Weber, G., and H. G. Drickamer. 1983. The effect of high pressure upon proteins and other biomolecules. *Q. Rev. Biophys.* 16:89–112.