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A critical role of protein-bound water in the catalytic cycle of cytochrome P-450 camphor

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The rates of NADH oxidation during the hydroxylation of camphor by cytochrome P-450$_{cam}$ were followed in the presence of co-solvents used to increase the osmotic pressure surrounding the protein-bound water. As a result, the measured V_{max} decreases independently of the perturbant tested. Roughly 28 molecules of water, involved during the catalytic cycle, are deduced from the variation of V_{max} as a function of osmotic pressure. These molecules, in part, could be those present in the cytochrome P-450$_{cam}$–putidaredoxin interface.

Cytochrome P-450$_{cam}$; Activity; Co-solvent; Osmotic pressure

1. INTRODUCTION

Cytochrome P-450$_{cam}$, a soluble globular hemeprotein, catalyses the hydroxylation of its physiological substrate, camphor, with high regiospecificity [1]. In substrate-free cytochrome P-450$_{cam}$, six water molecules, defined by X-ray cristallography, are present in the active site [2]. During the first step of the catalytic cycle, camphor binds close to the heme and excludes water from the heme pocket [3]. This process has been shown to control of the enzymatic activity of cytochrome P-450$_{cam}$ [4,5]. Of particular interest is to define the role that water could play in the following steps where cytochrome P-450$_{cam}$ is successively reduced, binds dioxygen, is reduced again to, finally, hydroxylate camphor [6–7].

The osmotic stress strategy has been used to measure the contribution of protein-bound water during the catalytic reaction. This elegant strategy, successfully used for other systems, revealed the crucial role that this thin layer of water plays to regulate the activity of proteins [8,9]. Co-solvents are added to the medium to increase the osmotic pressure surrounding the protein-bound water. The resulting perturbation can be reflected in characteristic parameters, such as the equilibrium constant and/or kinetic constant, which change as a function of osmotic pressure and are directly correlated to the number of molecules of water involved.

In this work the rate of NADH oxidation by the tertiary complex putidaredoxin reductase–putidaredoxin–cytochrome P-450$_{cam}$ during the hydroxylation of camphor was followed. Three osmolytes, glycerol, ethylene glycol and glucose, were used to increase the osmotic pressure.

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Abbreviation: NADH, β-nicotinamide adenine dinucleotide reduced.

2. MATERIALS AND METHODS

Cytochrome P-450$_{cam}$, putidaredoxin reductase and putidaredoxin were purified as previously described [10]. The assays were performed at 25°C in buffer (Tris-HCl 50 mM), with potassium chloride (50 mM) and camphor (1 mM) to insure proper saturation of the protein by the substrate whatever the co-solvent concentration may be. In the conditions choosen to measure the oxidation of NADH, any change of the concentration of one of the three proteins will change the measured rate. We kept the concentrations of putidaredoxin and putidaredoxin reductase, which transfert two electrons to cytochrome P-450$_{cam}$, constant at 4.2 μM and 1.75 μM, respectively. Only the concentration of cytochrome P-450$_{cam}$ was varied.

The oxidation of NADH was monitored at 340 nm by using a Uvikon 940 spectrophotometer (Kontron Instruments). An inverse plot of the rates of consumption of NADH as a function of the concentration of cytochrome P-450$_{cam}$ gives a maximum velocity for the oxidation of NADH, V_{max}, and an apparent constant, K, which corresponds to the concentration of cytochrome P-450$_{cam}$ at $V_{max}/2$.

The three osmolytes used to increase the osmotic pressure were ethylene glycol and glycerol (Janssen Chimica), and glucose (Sigma). These co-solvents were used without futher purifications. The proteins were mixed at low temperature to prevent denaturation and the formation of P-420, an inactive form of cytochrome P-450$_{cam}$. The osmotic pressure corresponding to each concentration of perturbant were calculated as previously described [8].

3. RESULTS AND DISCUSSION

The effect of co-solvents on the spectrum of ferric substrate-bound cytochrome P-450$_{cam}$ was used as a control. To minimize the effect of changes in viscosity

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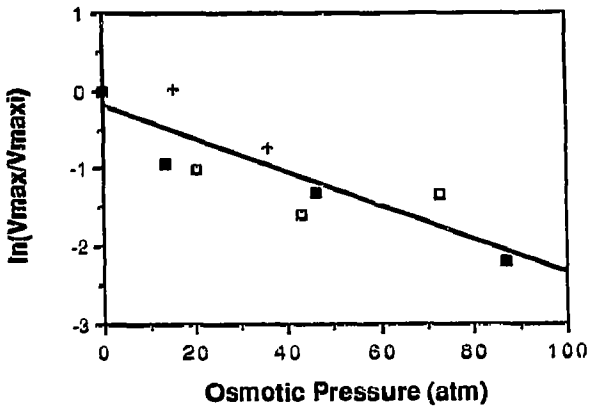

Fig. 1. Maximum velocity of NADH oxidation, V_{max}, as a function of osmotic pressure. The values obtained in co-solvents are plotted relative to the value in aqueous medium, V_{maxi}. (■) glycerol, (□) ethylene glycol, (+) glucose.

and dielectric constant, the concentration of co-solvents was kept below an osmotic pressure equal to 100 atm. No change in the Soret and the α, β bands is detected either in glycerol at 25% (weight percent) or ethylene glycol at 16% or glucose at 20%. The spectra are characteristic of the usual high spin form that camphor induces when it binds [1,11]. We also checked that the spectra of putidaredoxin reductase and putidaredoxin were not affected by the addition of co-solvents (results not shown).

The effect of increasing osmotic pressure on the rates of NADH oxidation is shown in Fig. 1. The experiments were performed first in glycerol until an osmotic pressure equal roughly to 100 atm was reached (glycerol 25%). The V_{max} relative to the value obtained in Tris-HCl buffer only, V_{maxi}, as a function of osmotic pressure is plotted. The increase in osmotic pressure results in a decrease of the rates of NADH oxidation. To check if this effect was really due to changes in the protein-bound water driven by osmotic pressure only, another co-solvent, ethylene glycol, was used. Within the experimental error, the data follow a linear relationship. Finally a quite different osmolyte, glucose, was tested. The results obtained strongly suggest that the effects observed are induced by changes in osmotic pressure.

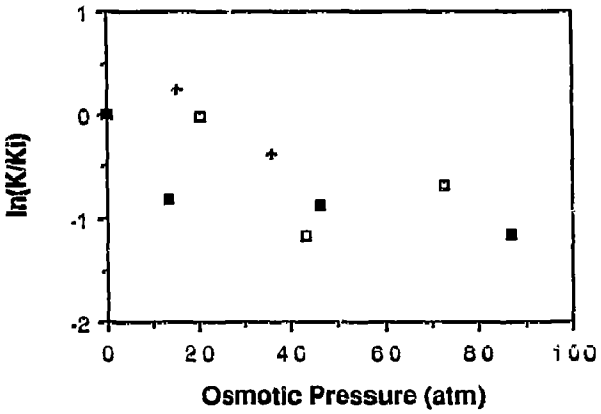

Fig. 2. Apparent constant, K, as a function of osmotic pressure. K corresponds to the concentration of cytochrome P-450$_{cam}$ where V_{max} is equal to $V_{max}/2$. The values obtained in co-solvents are plotted relative to the value in aqueous medium, K_i. (■) glycerol, (□) ethylene glycol, (+) glucose.

An estimation of the number of water molecules implicated in the reaction can be deduced from the slope of $\ln(V_{max}/V_{maxi})$ as a function of osmotic pressure equivalent to an activation volume change, ΔV^*. Assuming a partial molar volume of water equal to 18 ml/mol, the obtained value, 504 ml/mol, would correspond to 28 molecules of osmotically active water involved in the formation of the activated state. This number is actually a rough estimation. In this calculation we used the partial molar volume of free water. We did not take into account the change in partial molar volume when water binds to the protein.

Which step is affected by the increase in osmotic pressure? The catalytic cycle of cytochrome P-450$_{cam}$ is complex since three proteins are necessary to hydroxylate camphor. One could think that the decrease in NADH oxidation is due to an alteration of the interactions between cytochrome P-450$_{cam}$ and putidaredoxin in an electron transfer complex. This has been shown to be the rate-limiting step of the catalytic cycle [12]. A plot of K in co-solvents, relative to the value obtained in Tris-HCl buffer only, K_i, as a function of osmotic pressure is reported in Fig. 2. The value of K in co-solvents decreases with increasing osmotic pressure. If K is really an accurate reflection of K_m the decrease could be the result of the decreased V_{max} seen in Fig. 1. It should be recalled in the two-step Michaelis–Menten kinetic description that:

$$V_{max} = k_2 [E_T], \text{ and}$$

$$K_m = (k_{-1} + k_2)/k_1$$

where $[E_T]$ is the total concentration of enzyme and k_1, k_{-1}, k_2 are elementary kinetic constants. When the osmotic pressure is increased $[E_T]$ is constant but k_2 could decrease so that V_{max} and then K_m decrease. It is tempting to think that this change could be the result of modifications of the interfacial putidaredoxin–cytochrome P-450$_{cam}$ interactions. While there is not yet direct evidence for the presence of water at the interface of this key complex, this should not be neglected. In a recent model, the differential solvation of the C-terminal tryptophan residue of putidaredoxin is proposed as a mechanism which controls the association with cytochrome P-450$_{cam}$ and the subsequent electron transfer [13,14]. Any change of water activity around this residue could be responsible for the decreased V_{max}. This would confirm the major role of water in the association of these two proteins.

We cannot exclude that changes in osmotic pressure affect other steps. We have shown that the hydration degree of the heme pocket in turn determines the ability of cytochrome P-450$_{cam}$ to be reduced by its physiological partner [4], putidaredoxin, can be modified by osmotic pressure (results not published). This effect, probed by UV second derivative spectroscopy of Tyr-96, is quite drastic. The addition of polyethylene glycol 400 at 50% to substrate-free cytochrome P-450$_{cam}$ mimics the dehydration of the active site observed when camphor binds. The dehydrating effect of increasing osmotic pressure on the catalytically competent complex, camphor-bound protein, has also been observed.

The reduction of putidaredoxin by putidaredoxin reductase may also be altered. However, in this work, we checked, in the co-solvents tested, that putidaredoxin was normally reduced. It suggests that even in co-solvents the reduction of putidaredoxin by its physiological partner is not the rate-limiting step.

Recently, it was proposed that water could be directly involved in the mechanism of hydroxylation of camphor through a proton charge relay from the exposed surface to the deeply buried heme-iron (results not published). If true, any change of the protein-bound water by osmotic pressure could prevent this relay performing its function normally.

Obviously, water plays a critical role during the catalytic cycle of cytochrome P-450$_{cam}$. At least two major roles for water now appear. The first one during the binding step of the substrate, where movements of water in and out of the active site regulate the enzymatic activity, and the second one during the association of putidaredoxin with cytochrome P-450$_{cam}$. While the first one is well established, the other remains now to be clarified.

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