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Effects of monovalent cations on cytochrome P-450 camphor

Evidence for preferential binding of potassium

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Abstract

Binding of monovalent cations of increasing ionic radius to ferric cytochrome P-450$_{cam}$ was measured. Potassium has the highest affinity for the cation binding site observed in the X-ray crystallographic structure with $K_{d\,cat}$ = 12 mM, compared with the smaller cation lithium, ($K_{d\,cat}$ = 37 mM) and the larger cation cesium ($K_{d\,cat}$ = 20 mM). Coupling between cation binding and camphor binding is established by the observation of a linear relationship between the corresponding binding free energies. Potassium binding favours a conformational change of tyrosine 96 which increases the affinity of the protein for camphor and fully dehydrates the active site.

Key words: Cytochrome P-450$_{cam}$; Cation binding; Camphor binding; Spin transition.

1. Introduction

Cytochrome P-450$_{cam}$ is a hemoprotein from *Pseudomonas putida* that catalyzes the formation of 5-exo-hydroxycamphor from camphor. The X-ray crystallographic structure of the protein has been established in both the substrate-free [1] and substrate-bound forms [2,3] as well as with other ligands [4–7]. Camphor binds between two well defined structural domains (helix-rich *domain* composed of helices C–L and the random coil β-sheet *domain*), in a buried active site with no direct access to the solvent. In the substrate-free protein six water molecules are present in the active site, one of which is coordinated to the heme iron as its sixth ligand in the oxidized hemoprotein. Camphor binding induces a spin transition of the heme iron from low spin to high spin and the displacement of the water molecules from the heme pocket to the solvent. A cation binding site was proposed at the position of water 515 involving the carbonyl backbones of glutamate 84, glycine 93, glutamate 94, tyrosine 96 as well as water molecules 517 and 584 in an octahedral geometry [3]. A hydrogen bond between the hydroxyl of tyrosine 96 and the ketone group of camphor provides an additional link between the enzyme and the substrate [2,8].

In cytochrome P-450$_{cam}$ the affinity for camphor increases in the presence of potassium [9,10]. This cation also influences the spin equilibrium of the substrate-bound protein [10–12] which is controlled by water access to the active site [13] with the resultant change in polarity in the substrate binding pocket, as quantitated by second derivative UV spectroscopy [14]. Binding of potassium to the cation binding site would stabilize an otherwise unfavourable B′ helix. This causes a conformational change at tyrosine 96 resulting in the removal of water from the active site and an increase in the affinity for the substrate. The two structural domains of the protein are then tightly linked together by camphor and the structure becomes more rigid as suggested by the increased stability of the protein when submitted to hydrostatic pressure [15–17]. Removal of the tyrosine 96–camphor hydrogen bond results in an alteration of several physicochemical features of the system, including water occupancy of the active site [8], hydroxylation regiospecificity [8], stability versus hydrostatic pressure [17], and potassium binding [10].

The choice of potassium as the candidate for the cation binding site is based only on the experimental observations mentioned above. In the crystallographic structure this cation could not be seen and in fact it was never established if there was a specifity for this cation. We were interested in determining whether other cations induce similar effects and whether there is a simple relationship between the observed effects and the size of the cation. We also wished to test the proposal that a coupling exists between cation binding and camphor binding [3,10]. To address these questions, the effects of

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Abbreviations: Cytochrome P-450$_{cam}$, cytochrome P-450 camphor; Li^+, lithium; Na^+, sodium; K^+, potassium; Rb^+, rubidium; Cs^+, cesium; Mg^{2+}, magnesium; Ca^{2+}, calcium; $K_{d\,cat}$, apparent dissociation constant for the cation; $K_{d\,cam}$, apparent dissociation constant for camphor; ΔG_{hyd}, cation hydration free energy; ΔG_{cam}, camphor binding free energy; ΔG_{cat}, cation binding free energy; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate.

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monovalent cations of increasing ionic radius ($Li^+ < Na^+ < K^+ < Rb^+ < Cs^+$) were examined.

2. Materials and methods

The camphor-bound form of cytochrome P-450$_{cam}$ was generated and purified as previously described [18,19] and stored at −80°C. Substrate-free protein was obtained by passing a sample of pure concentrated protein through a Sephadex G-25 column which had been previously equilibrated at 4°C with the buffer used for the experiments, 50 mM Tris-HCl (Sigma), pH 7 at 20°C.

The apparent dissociation constant for the monovalent cation, $K_{d\,cat}$, was deduced from the variation of the high spin fraction as a function of monovalent cation concentration as described elsewhere [10]. The percentage of high spin was calculated according to Jung's procedure [20]. The experiments were done on the substrate-bound protein since the substrate-free protein is not sensitive to cations (unpublished results). Camphor (Merck) was added at saturating concentration, 400 μM. The apparent dissociation constant for camphor, $K_{d\,cam}$, was determined at two wavelengths [8], 417 nm and 392 nm, which correspond to the absorption peaks of the low spin and high spin forms, respectively. The measures were performed on a Uvikon 940 spectrophotometer. The temperature was maintained at 20 ± 0.5 °C with a Huber HS 40 circulating bath. The free energies of hydration reported in Table 1 are from reference [21].

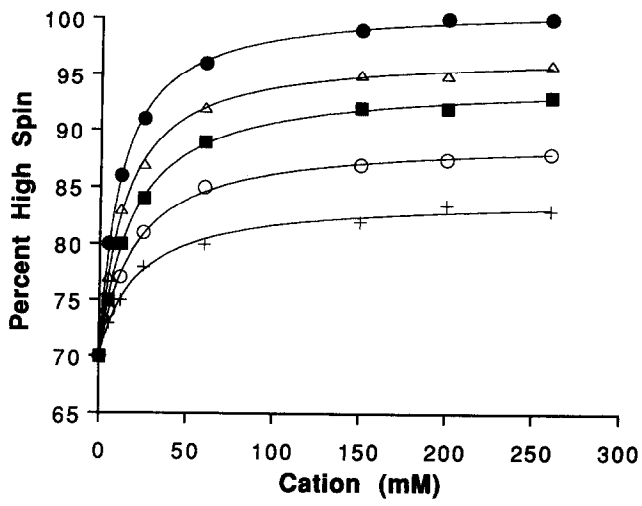

Fig. 1. Percentage of high spin as a function of the concentration of cation. (●) K^+, (△) Rb^+, (■) Cs^+, (○) Na^+, (+) Li^+. Camphor is present at saturating concentration, 400 μM.

3. Results and discussion

Binding of cations is a common feature of proteins. For instance, protein kinase C is activated by calcium [22]. In the glycolysis chain reaction, pyruvate kinase needs potassium and magnesium to convert phosphoenol pyruvate and ADP to pyruvate and ATP [23].

In this work binding of monovalent cations of increasing ionic radius to camphor-bound cytochrome P-450$_{cam}$ was measured. The influence of these cations on the spin equilibrium displayed by the protein was investigated first. The results are presented in Fig. 1. In the absence of cation, camphor shifts the spin equilibrium from 5% high spin to 70% high spin which is the starting point of the plot for the cation dependence. This percentage increases with increasing concentrations of all cations tested but only potassium induces a complete transition to virtually 100% high spin, corresponding to a totally dehydrated active site. It is evident that this cation induces the most complete conformational change of tyrosine 96, decreasing water access to the heme pocket by the proposed 'polar route' [10]. The observed effects cannot be attributed to changes in the ionic strength, since the maximum percent high spin is dependent upon the identity of the cation. Futhermore we have shown that spermine, a polycation too large to bind to the cation binding site, shifts the spin equilibrium to only 75% high spin [24], suggesting that ionic strength effects play only a small role in the transition. The apparent dissociation constant, $K_{d\,cat}$, was determined from these plots for each cation, and these values are reported in Table 1. Potassium is the cation which binds the best to cytochrome P-450$_{cam}$ with $K_{d\,cat}$ equal to 12 ± 1 mM in agreement with previous results [10].

In Fig. 2 the $K_{d\,cat}$ for each cation and the percentage of high spin form induced at saturation of cation are plotted as a function of the free energy of hydration of the cation (ΔG_{hyd}). As reported in Table 1 ΔG_{hyd} varies inversely with the ionic radius of the cation. The smallest cation, lithium, has the largest free energy of hydration, while cesium, the cation with the largest ionic radius, has the smallest value for ΔG_{hyd}. From this figure it is evident that the cation which binds the best to cytochrome P-450$_{cam}$, potassium, is also the one which induces the largest shift in spin equilibrium (to 99% high spin). Since the

Table 1
Cations and camphor binding parameters

	Cs^+	Rb^+	K^+	Na^+	Li^+
Cation radius (Å)	1.69	1.48	1.33	0.95	0.60
ΔG_{hyd} (kcal/mol)	− 47	− 51	− 55	− 72	− 98
$K_{d\,cat}$ (mM)	20 ± 2	15 ± 1	12 ± 1	24 ± 2	37 ± 3
$K_{d\,cam}$ (μM)					
at 60 mM cation	6.0 ± 0.3	3.8 ± 0.1	2.4 ± 0.2	7.4 ± 0.3	10.5 ± 0.5
at 260 mM cation	3.4 ± 0.3	2.2 ± 0.2	1.6 ± 0.1	4.2 ± 0.2	6.0 ± 0.4
% High spin at 260 mM cation	93	96	99	88	83

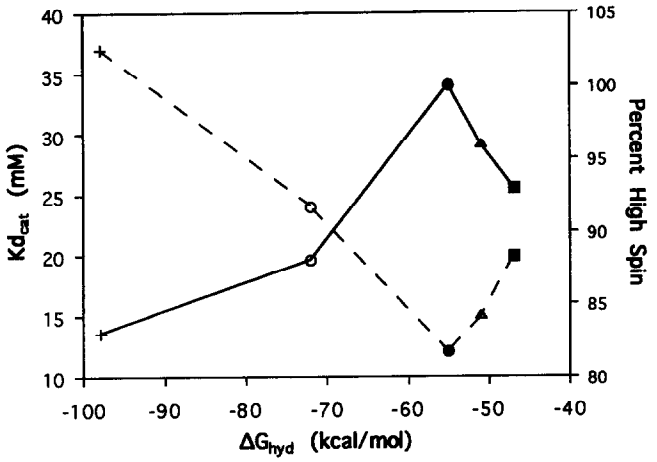

Fig. 2. Percentage of high spin and dissociation constant for the cation, $K_{d\,cat}$, as a function of the hydration free energy of the cation, ΔG_{hyd} [21]. (—) percent high spin, (- - - -) $K_{d\,cat}$. (+) Li^+, (○) Na^+, (●) K^+, (△) Rb^+, (■) Cs^+.

dissociation constant is an apparent constant which takes into account the hydration of the cation and its intrinsic affinity for the binding site, the cation with the lowest apparent dissociation constant is not necessarily the one which fits the best into the anionic cage. Molecular simulations of complexes between dibenzocrown ethers and alkali cations [25,26] have shown that when solvation effects are not considered the stability of the Na^+ complexes is greater than that of the corresponding K^+, Rb^+ and Cs^+ complexes. However, when solvation is taken into account, the overall binding affinity results from a competition between the affinity of the cation for the solvent and its affinity for the binding site. Thus, the preference for Na^+ over K^+ in these model systems is not enough to compensate for the large preference of the solvent for Na^+ over K^+, and the stability of the K^+-crown ether complexes becomes greater than the Na^+ complexes. In cytochrome P-450_{cam}, potassium presumably represents the best compromise between intrinsic affinity for the binding site and free energy of solvation. Changing from potassium to larger or smaller cations results in a decrease in the observed effects upon spin equilibrium and cations binding. However, cations with larger ionic radii (rubidium and cesium) than potassium are more efficient than smaller ones (sodium and lithium) in binding to cytochrome P-450_{cam} and shifting the spin equilibrium towards the high spin forms. All the atoms forming the octahedral anionic cage are within 3 Å of solvent 515 [3]. This is a rather large cation binding site for small cations such as sodium compared with known models [27] and thus could explain why large cations are more effective at inducing the conformational change of tyrosine 96 that enhances the dehydration of the active site. In addition, the smaller hydration free energy of rubidium and cesium compared with sodium and lithium would also favour binding to the protein.

Interestingly, in cytochrome P-450_{cam} the preference for K^+ over all other cations is not as high as was observed in the valynomicin ionophore which binds K^+ 1,000-fold better than Na^+ [28]. The diameter of the internal cavity of this ionophore closely fits K^+. Futhermore, its rigid structure cannot accomodate Na^+ as well. In cytochrome P-450_{cam}, the large size of the anionic cage compared with the size of the cations and the high degree of flexibility in this region as suggested by the temperature factor [3] and molecular dynamic simulations [29,30] could explain why this cation binding site can also efficiently accomodate other monovalent cations and even divalent cations such as Mg^{2+} (ionic radius 0.65 Å) and Ca^{2+} (ionic radius 0.99 Å) [9]. Although binding of magnesium and calcium to the protein are even tighter than potassium (data not shown), in agreement with previous work [9], these divalent cations do not induce a complete transition to high spin and do not increase the affinity of the enzyme for camphor as well as potassium does. The extent of these divalent cations' influence on spin equilibrium and camphor binding is similar to that of monovalent cations with comparable ionic radii. This suggests that induction of the conformational change at tyrosine 96 does not depend upon the valence of the cation but rather upon its size, although the valence does play a key role in binding to the anionic cage.

The binding of camphor to cytochrome P-450_{cam} was measured at saturating (260 mM) and non saturating (60 mM) concentrations of cation. The results are presented in Table 1 and Fig. 3. Under both conditions, the tightest binding of camphor is obtained with potassium. $K_{d\,cam}$ is equal to 1.6 ± 0.1 μM with potassium concentration at 260 mM while with lithium at the same concentration $K_{d\,cam}$ is equal to 6.0 ± 0.4 μM. Without cation, $K_{d\,cam}$ is equal to 12 μM. For cations other than potassium, the affinity for camphor is higher with larger cati-

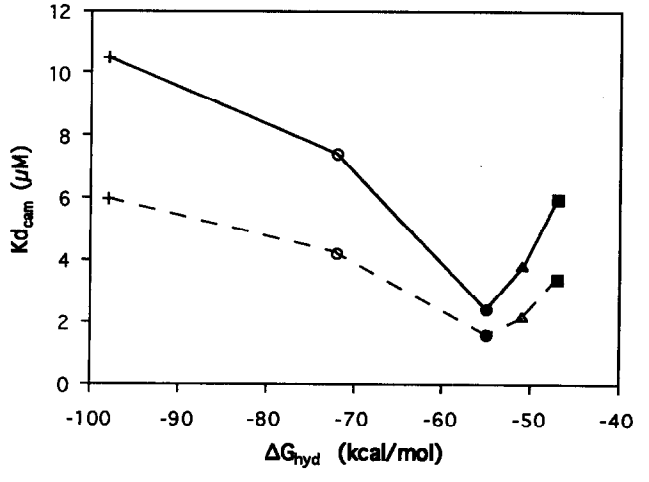

Fig. 3. Dissociation constant for camphor, $K_{d\,cam}$, at two concentrations of cation as a function of the free energy of hydration of the cation, ΔG_{hyd} [21]. (—) 60 mM, (- - - -) 260 mM. (+) Li^+, (○) Na^+, (●) K^+, (△) Rb^+, (■) Cs^+.

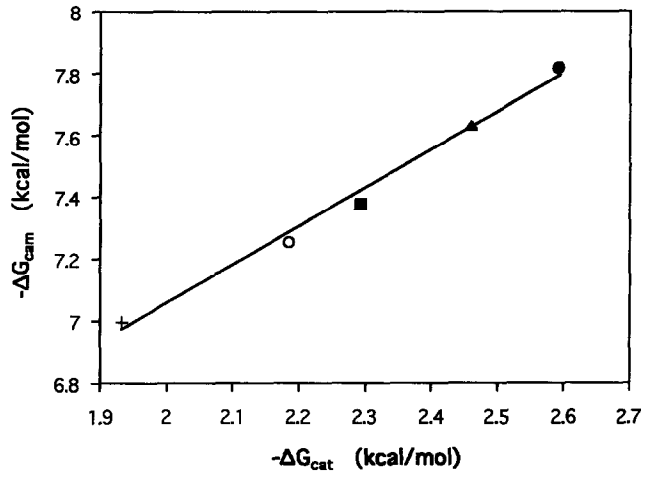

Fig. 4. Binding free energy of camphor, $\Delta G_{cam} = -RT \ln(1/K_{d\,cam})$, as a function of the binding free energy of the cation, $\Delta G_{cat} = -RT \ln(1/K_{d\,cat})$. (+) Li^+, (○) Na^+, (■) Cs^+, (△) Rb^+, (●) K^+. Cations are at saturating concentration, 260 mM.

ons (rubidium and cesium) than with smaller ones (sodium and lithium).

It has been proposed but never demonstrated that cation binding and camphor binding are coupled. We report in Fig. 4 the camphor binding free energy, $\Delta G_{cam} = -RT \ln(1/K_{d\,cam})$, as a function of the cation binding free energy, $\Delta G_{cat} = -RT \ln(1/K_{d\,cat})$. The linear relationship clearly demonstrates the existence of the coupling between cation binding and camphor binding. As previously shown [10], removal of tyrosine 96 by site-directed mutagenesis affects both potassium binding and camphor binding. This result suggests that tyrosine 96 is responsible for the coupling between cation binding and camphor binding.

In conclusion, despite the ability of cytochrome P-450_{cam} to bind to several different cations, potassium is the one that induces the largest effects. Its binding to the octahedral anionic cage induces a conformational change of tyrosine 96 which increases the affinity for camphor and reduces its mobility in the heme pocket. Water molecules are expulsed as seen by the complete transition to high spin. The redox potential of the protein is then favorable for reduction by putidaredoxine [31,32]. Camphor hydroxylation at the 5-position is highly regio-stereospecific compared with mobile substrates [33] or a mutant in which the hydrogen bond between tyrosine 96 and substrate was suppressed [8,33].

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