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Archives of Biochemistry and Biophysics 455 (2006) 154–162
ABB
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Effect of alcohols on binding of camphor to cytochrome P450cam: Spectroscopic and stopped flow transient kinetic studies

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Received 8 August 2006, and in revised form 10 September 2006
Available online 29 September 2006

Abstract

Addition of alcohols to cytochrome P450cam (CYP101) was shown to release the substrate camphor from the heme pocket of the enzyme. The release of the substrate was found to be caused both due to increased solubility of the substrate in solution in presence of alcohol and due to change in the tertiary structure of the active site of the enzyme. The far-UV CD and near-UV CD spectra reveal that addition of alcohols to cytochrome P450cam cause a small change in the secondary structural elements but a significant change in the tertiary structural organization of this enzyme. The CD spectra at the heme region at various concentrations of alcohols indicate a substantial change in the tertiary structural organization around the heme moiety too. The equilibrium constant associated with the binding of camphor to Cyt P450cam is strongly dependent on the concentration of alcohols and the corresponding free energy associated with the binding is found to scale linearly with the concentration of alcohols. Kinetic experiments on binding of camphor to Cyt P450cam show that both k_{on} and k_{off} rate constants are strongly affected by addition of alcohols suggesting that alcohol expel camphor out of the heme cavity of Cyt P450cam by affecting tertiary structure of Cyt P450cam as well as by modifying the solubility properties of camphor in aqueous medium.
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Keywords: Alcohol induced unfolding; Camphor binding; Cyt P450cam; Camphor binding kinetics; Effect of alcohol on substrate binding

The cytochrome P450s form an important superfamily of heme containing mono-oxygenases, capable of site specifically hydroxylating a variety of hydrophobic substrates including drugs, xenobiotics and steroid hormones [1]. The substrate-binding site of these enzymes resides close to the heme active center that is completely encapsulated by the protein matrix. Even small conformational changes or perturbations near the active site can cause slips in the catalytic function of the enzyme leading to leakage of active oxygen resulting in the formation of hydrogen peroxide or superoxide instead of the usual mono-oxygenation product during the redox catalytic cycle [2]. The cytochrome P450 (Cyt P450cam, E.C.: 1.14.15.1) obtained from soil bacterium, *Pseudomonas putida* is one of the well-studied members of this superfamily, which carries out the hydroxylation of camphor specifically at the 5-exo position during its metabolic cycle to enable the bacterium to utilize camphor as its carbon source [1,3,4]. The ferric ion of heme in Cyt P450cam [5–10] is low-spin six-coordinated in absence of camphor with fifth axial ligation to the sulphur of Cys357, and a water molecule is coordinated at the sixth coordination position of the metal ion [1].

Binding of substrate to Cyt P450cam involves at least two sequential steps [11–15]. The first one is the substrate entry, which involves the interaction of the substrate with side chains of Tyr96, Phe87 etc., [1,3] that decides the specificity and the correct orientation of the substrate in the heme pocket. The second step involves the knocking out of water molecule from the sixth axial position of the heme leading to the spin transition of the iron [1]. Apart from forming the enzyme-substrate complex, binding of camphor is also important for the formation of compact structure of

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0003-9861/$ - see front matter © 2006 Published by Elsevier Inc.
doi:10.1016/j.abb.2006.09.007

the protein interior near the heme active-site [16]. Presence of camphor in the medium is believed to help in proper folding of this enzyme and, therefore, the purification of recombinant Cyt P450cam is often carried out in presence of camphor [17–20].

The thermodynamic stability of the protein structure can be quantified from equilibrium unfolding studies induced by heat or by chemical denaturants such as urea and guanidine hydrochloride (GdnCl)[1] [21,22]. Unfolding of Cyt P450cam and few other Cyt P450s by pressure [23] or denaturants [22,24–26] have earlier been studied. Recent unfolding studies by us [22] on Cyt P450cam by urea and temperature indicated the presence of at least two sequential intermediates in the unfolding pathway of this enzyme both in the presence as well as in the absence of camphor. Moreover, presence of camphor in the medium was found [22] to increase the midpoint denaturant concentrations (C_m) associated with the unfolding of Cyt P450cam by urea. Apart from chemical denaturants such as urea and guanidine hydrochloride, alcohols are also extensively used as denaturing co-solvents [27–29]. Although ethanol has been shown to enhance expression of certain P450 isoenzymes in vivo [30] as well as in vitro [31], alcohols can also perturb the structure and there by affect the function of these enzymes [29,32,33]. However, so far no detailed molecular level studies have been carried out to understand the effect of alcohols on the binding of substrates to the cytochrome P450s. In the present study, we have investigated the structure and substrate-binding properties of Cyt P450cam in presence of different alcohols. We show that the addition of alcohol to camphor-bound Cyt P450cam causes expulsion of substrate from the heme cavity without actually affecting the overall secondary structural components of the enzyme but causing significant change to the tertiary structure of the active site of the enzyme. The effect of alcohol on the structure and substrate-binding properties of the enzyme was found to depend on the nature of the alcohol.

Materials and methods

Over-expression and purification of Cyt P450cam

DEAE Sepharose, Q Sepharose, Sephadex G-10, and urea were purchased from Roche chemicals. Camphor was purchased from Sigma, USA and all other chemicals were of analytical grade. Cyt P450cam was over-expressed in BL21 (DE3) *Escherichia coli* strain and purified using already reported protocol [17,22,34]. The purified enzyme was stored at −20°C in 40 mM potassium phosphate buffer (pH 7.4 containing 1 mM of camphor) containing 40% glycerol, after passing through a 0.22 μm membrane filter. Camphor-free Cyt P450cam was prepared by passing the protein sample through a Sephadex G-10 column pre-equilibrated with 25 mM Tris, pH 7.5 (at 4 °C). The concentration of Cyt P450cam was determined [17] using the heme absorbance at 392 nm for camphor-bound Cyt P450cam ($\varepsilon_{392} = 102\ mM^{-1}\ cm^{-1}$) and at 417 nm ($\varepsilon_{417} = 115\ mM^{-1}\ cm^{-1}$) for camphor-free Cyt P450cam. Here, unless otherwise specified, all the protein samples and camphor stock solutions were prepared in 40 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl.

Alcohol induced expulsion of camphor from the heme cavity

We have used three different alcohols viz. methanol, ethanol and isopropanol. To study the effect of various alcohols on absorption spectrum at the Soret region, ~5 μM of Cyt P450cam in presence of ~200 μM of camphor was titrated with different alcohols and the corresponding absorption spectra of Cyt P450cam at different concentrations of alcohols were recorded on Schimadzu UV2100 spectrophotometer with a path length of 1 cm. All the experiments were carried out at room temperature (i.e. 298 K). The secondary structure of camphor-bound Cyt P450cam was studied by taking the far-UV CD spectra (190–260 nm) of ~3 μM of Cyt P450cam in presence of ~200 μM of camphor at different alcohol concentrations using a JASCO 810 spectropolarimeter with a path length of 1 mm. The near-UV and visible CD spectra of Cyt P450cam (~25 μM) was studied at different concentrations of various alcohols using a cuvette with path length of 1 cm. The equilibrium constant associated with the binding of camphor to Cyt P450cam at different concentrations of alcohols were determined from the spectra obtained from the titration of ~5 μM of camphor-free Cyt P450cam with ~500 μM stock solution of camphor (here, the protein as well as the camphor solutions contain the corresponding concentrations of alcohols) in presence of 100 mM KCl.

The equilibrium: Cyt P450cam + Camphor $\underset{K_d}{\rightleftharpoons}$ Cyt P450cam-camphor can be analysed using a generalized equation taking $K_d = (p_0 - p_B)(C_0 - p_B)/p_B$, where K_d (M) is the dissociation constant of the camphor-bound Cyt P450cam, p_0 (M), C_0 (M) are initial concentrations of the enzyme and camphor, respectively, and p_B is the concentration of substrate-bound form of the enzyme. Considering that the absorbance at 417 nm (as well as at 391 nm) are contributed by both the substrate free and substrate bound forms of the enzyme, a generalized non-linear equation can be derived to correlate the observed absorbance to the concentration of camphor (C_0) during the titration experiment.

The equilibrium constants associated with the binding of camphor to Cyt P450cam were determined from the spectral cross-sections at 417 nm. The absorbance at 417 nm (A_{417}) is given as: $A_{417} = \varepsilon^{b}_{417}.p_B + \varepsilon^{f}_{417}.p_f$, where ε values are the corresponding molar extinction coefficients for camphor bound (p_B) and camphor-free (p_f) Cyt P450cam at 417 nm. Since camphor does not have absorption in this spectral region and there is a well-defined isosbestic point in the absorption spectra of the titration of the enzyme by camphor at each concentration of the alcohol, one can derive the following generalized nonlinear equation for variation of absorbance at 417 nm with camphor concentration (Eq. (1)).

$$A_{417} = \varepsilon^{b}_{417} p_0 - \left(\varepsilon^{f}_{417} - \varepsilon^{b}_{417}\right)\left(\frac{[(p_0 + C_0) + K_d] - \sqrt{[(p_0 + C_0) + K_d]^2 - 4p_o C_0}}{2}\right) \quad (1)$$

Here, C_0 (M) is the total concentration of camphor in the enzyme solution at any given point during the titration. Non-linear least squares fit to the observed titration data (A_{417} vs. C_0) with the Eq. (1) gives the values of K_d value. From the inverse of K_d one can obtain the equilibrium constant associated with the binding of camphor to cyt P450cam as $K_b = 1/K_d$.

The rate constants for binding of camphor in presence of different concentrations of alcohols were measured by HiTech-SF61MX stopped flow spectrometer. The temperature was maintained at 298 K using a water jacketed temperature controller. The following relationship was used to calculate k_{on} ($mol^{-1}\ s^{-1}$) and k_{off} (s^{-1}) of binding of camphor to Cyt P450cam.

$$k^{obs}_{app} = k_{on} C_0 + k_{off} \quad (2)$$

For kinetic experiments ~20 μM of camphor-free Cyt P450cam treated with different concentrations of alcohols was rapidly mixed with a series of camphor concentrations (also containing the corresponding concentrations of alcohols) and the time evolutions of decreasing in the absorbance at 417 nm and increasing in the absorbance at 392 nm were recorded. The

[1] *Abbreviations used:* CYP101, cytochrome P450cam; GdnCl, guanidine hydrochloride.

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