The thermodynamics and kinetics of electron transfer in the cytochrome $P450_{cam}$ enzyme system

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Abstract In anaerobic environments the first electron transfer in substrate-free $P450_{cam}$ is known to be thermodynamically unfavourable, but in the presence of dioxygen the reduction potential for the reaction shifts positively to make electron transfer thermodynamically favourable. Nevertheless a slower rate of electron transfer is observed in the substrate-free $P450_{cam}$ compared to substrate-bound $P450_{cam}$. The ferric haem centre in substrate-free $P450_{cam}$ changes from six co-ordinate to five co-ordinate when reduced whereas in substrate-bound $P450_{cam}$ the iron centre remains five co-ordinate in both oxidation states. The slower rate of electron transfer in the substrate-free $P450_{cam}$ is therefore attributed to a larger reorganisation energy as predicted by Marcus theory.

Key words: Monooxygenase; P450; Electron transfer; Redox potential; Oxygen binding

1. Introduction

The cytochrome P450 family of monooxygenase enzymes has been much studied over the last 30 years. The enzymes catalyse the hydroxylation, or more correctly oxygen atom insertion, of their substrates [1]. Bacterial cytochrome $P450_{cam}$ ($P450_{cam}$) from *Pseudomonas putida* has been one of the most studied of the P450 enzymes [2,3]. $P450_{cam}$ receives two electrons from its redox partner, putidaredoxin, in two separate steps during the catalytic cycle as shown in Fig. 1.

The two separate electron transfer steps have been shown to be the slowest in the $P450_{cam}$ catalytic cycle [1]. Since both are bimolecular reactions the observed rates depend on the $P450_{cam}$:putidaredoxin ratio. The activity of $P450_{cam}$ is assessed in vitro with typically greater than eight-fold excess of putidaredoxin such that the first electron transfer is the rate-limiting step for the overall catalytic cycle [4]. Therefore much attention has been focused on the factors controlling the rate of this step within the context of both understanding the activity of the enzyme per se and the oxidation of unnatural substrates for biotechnological applications.

The rate of the first electron transfer from putidaredoxin to $P450_{cam}$ is considerably slower in the absence of the substrate camphor compared to electron transfer to the camphor-bound enzyme [1]. When camphor binds to $P450_{cam}$ a large positive shift in what is referred to in the electrochemical literature as the formal potential [5,6], $E^{0'}$, and in the biological literature as the midpoint potential [7], E_m, occurs and this has been offered as a reason for the differences in the respective rates. The formal potentia1 (at pH 7.0) of camphor-bound $P450_{cam}$ has been reported as being approximately −170 mV whereas that of the substrate-free enzyme has been reported as ranging from −270 to −330 mV [8,9]. The formal potential of putidaredoxin is −235 mV [10] and therefore the reduction of substrate-free $P450_{cam}$ should be thermodynamically unfavourable. The variation in the literature value for the substrate-free formal potential probably reflects the experimental uncertainties associated with the dye photoreduction method used to determine the reduction potential, particularly when the dye reduction potential is significantly different to the enzyme formal potential [7].

Whilst the exact values of the formal potentials for substrate-free and camphor-bound forms of $P450_{cam}$ remain to be unambiguously determined, it seems that a positive shift in potential occurs upon substrate binding. This potential shift is a common feature of P450 systems, and is generally proposed to transform the electron transfer reaction from one that is thermodynamically unfavourable to one that is thermodynamically favourable. Hence it is speculated that the potential shift following substrate binding is nature's way of preventing the futile cycling of electrons [11].

The potential shift following substrate binding has been attributed to the ferric spin-state shift that accompanies substrate binding. In the substrate-free form of $P450_{cam}$ the ferric haem centre is six co-ordinate and predominantly low spin with a water molecule co-ordinated in the distal position. In the substrate-bound form the ferric haem centre loses the water molecule, becoming five co-ordinate and predominantly high spin. The haem iron in ferrous $P450_{cam}$ is five co-ordinate and high spin in both its substrate-free and substrate-bound forms.

2. The effect of dioxygen binding on the reduction potential

Some factors in the thermodynamics and kinetics of the first electron transfer step, which initiates the catalytic cycle and determines the overall turnover rates, appear to have been overlooked. A common misconception in the literature seems to be that judgements about magnitude of the driving force for the first electron transfer from putidaredoxin to $P450_{cam}$ can be made without consideration of the other steps in the $P450_{cam}$ catalytic cycle. Discussions about the driving force for the first electron transfer usually only focus on the values of the formal potentials given above. Perhaps these misconceptions stem from the consideration of the electron transfer reaction in molecular terms when the formal potentials are properties associated with, and measured from, bulk properties. Even in the absence of any coupled reactions no judgement about whether a reaction is thermodynamically favourable can be made based solely on the formal potential. The

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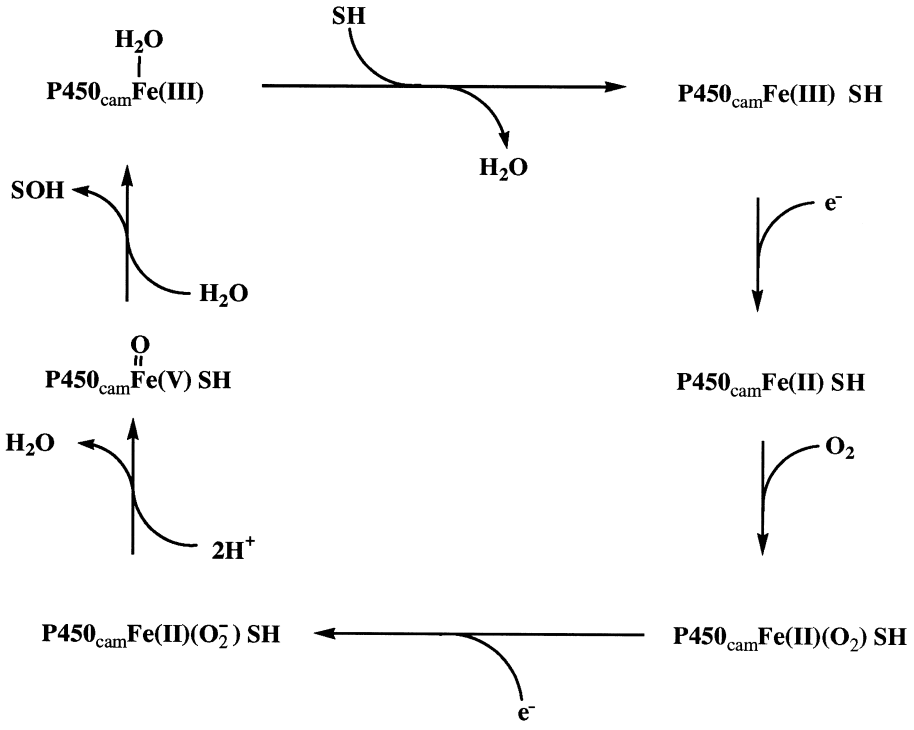

Fig. 1. The cytochrome $P450_{cam}$ catalytic cycle. The representations of the intermediates are for formal electron counting purposes and not the actual electronic structure of the species themselves.

concentrations, or more strictly speaking the activities, of both the reactants and products need to be taken into account as given by the Nernst equation. The electron transfer from putidaredoxin is preceded by the transient binding of putidaredoxin to the protein for which we can write the following reactions:

$$P450_{cam}Fe(III) + Pd^{Red} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P450_{cam}Fe(II) + Pd^{Ox} \quad (1)$$

$$E = E^{0'} + \frac{RT}{F}\ln\left(\frac{[P450_{cam}Fe(III)]}{[450_{cam}Fe(II)]}\frac{[Pd^{Red}]}{[Pd^{Ox}]}\right) \quad (2)$$

If chemical reactions are coupled to an electron transfer step its reduction potential, i.e. the value of E in Eq. 2, will be altered. For example ligand binding to a reactant lowers the reduction potential while ligand binding to a product increases it. In the $P450_{cam}$ catalytic cycle the reduction of the ferric haem by putidaredoxin, Eq. 1, is followed by the rapid binding of dioxygen to the ferrous haem [12].

$$P450_{cam}Fe(II) + O_2 \xrightarrow{k_2} P450_{cam}Fe(II)\cdot O_2 \quad (3)$$

Therefore the presence of dioxygen increases the reduction potential for Eq. 1. The rate of oxygen binding to ferrous $P450_{cam}$, k_2, is typically orders of magnitude faster than the reverse electron transfer reaction, k_{-1}. For both substrate-bound and substrate-free $P450_{cam}$ we can therefore apply the steady-state assumption to the concentration of the ferrous form and Eq. 2 can thus be rewritten as

$$E = E^{0'} + \frac{RT}{F}\ln\left(\frac{k_2[O_2] + k_{-1}\,[Pd^{Ox}]}{k_1\,[Pd^{Ox}]}\right) \quad (4)$$

Since $k_2 \gg k_1, k_{-1}$ and the ambient concentrations of dioxygen in the cytoplasm ($\sim$mM) are larger than those of putidaredoxin we can approximate $k_2[O_2]+k_{-1}[Pd^{Ox}] \cong k_2[O_2]$ and rewrite Eq. 4 as

$$E \cong E^{0'} + \frac{RT}{F}\ln(k_2[O_2]) - \frac{RT}{F}\ln\,(k_1[Pd^{Ox}]) \quad (5)$$

It follows from Eq. 5 that the reduction potential of both substrate-bound and substrate-free $P450_{cam}$ will be expected to increase in the presence of dioxygen by approximately 60 mV per decade increase in dioxygen activity. This increase in the reduction potential is likely to be sufficient to make electron transfer from reduced putidaredoxin to *substrate-free* $P450_{cam}$ thermodynamically favourable. These thermodynamic arguments suggest that electrons will be cycled in the absence of substrate, whether futile or not.

3. The rate of the first electron transfer

Of course no conclusions can be drawn about the kinetics of a reaction based simply on an observation of favourable thermodynamics. There are numerous examples in nature of a reaction being extremely thermodynamically favourable but kinetically very slow so a positive shift in the reduction potential does not in itself completely explain the more rapid kinetics of the electron transfer in the substrate-bound complex.

Given that the thermodynamics of Eq. 1 become more favourable in the presence of dioxygen we must examine why the NADH turnover rate is slow in substrate-free $P450_{cam}$. Since the rate of dioxygen binding is much faster than the rate of the first electron transfer, $k_2 \gg k_1$, the ferrous haem is continually being consumed and the turnover rate of the

catalytic cycle is controlled by the magnitude of k_1. We can use the semi-classical Marcus equation [13] for the electron transfer rate to consider what factors may influence the magnitude of k_1:

$$k_1 \propto \exp\left(-\frac{\lambda + \Delta G_1^0)^2}{4\lambda k_B T}\right) \quad (6)$$

where ΔG_1^0 is the change in standard free energy of Eq. 1, λ is the reorganisation energy, the sum of both inner sphere and outer sphere components.

In substrate-free $P450_{cam}$, ΔG_1^0 is approximately 0.1 eV more positive (less favourable) than for the substrate-bound form, suggesting a slower rate of electron transfer to substrate-free $P450_{cam}$ if the reorganisation energies were the same for both reactions. However, the transition from the oxidised substrate-free $P450_{cam}$ from a six co-ordinate complex to a five co-ordinate complex upon reduction is likely to result in a larger inner sphere reorganisation energy, λ_{inn}, than in substrate-bound $P450_{cam}$ which is five co-ordinate in both oxidation states. Assuming similar values for the outer sphere reorganisation energy, λ_{out}, the total reorganisation energy, λ, for the electron transfer between putidaredoxin and substrate-free $P450_{cam}$ is likely to be of the order of a few tenths of an eV larger than electron transfer between putidaredoxin and substrate-bound $P450_{cam}$. Therefore, relative to substrate-bound $P450_{cam}$, the substrate-free form has both a less favourable ΔG_1^0 *and* larger λ from which, according to Marcus theory, a lowering of the magnitude of k_1 is expected.

The magnitude of λ will be of the order of 0.3–0.9 eV which is 5–10 times the reported difference in ΔG_1^0 between substrate-bound and substrate-free $P450_{cam}$ in Eq. 1 meaning that λ is the dominant factor affecting the electron transfer rate. In fact, based on Eq. 6, because of the expected large increase in λ, a significant decrease in the reduction rate of substrate-free $P450_{cam}$ would be observed regardless of whether a difference in the formal potential for Eq. 1 existed between the substrate-bound and substrate-free forms.

4. Conclusions

The presence of dioxygen increases the reduction potential for the first electron transfer step. Under typical biological conditions the increase will be sufficient to make the first electron transfer in substrate-free P450 thermodynamically favourable.

The reorganisation energy barrier for the first electron transfer step is likely to be larger for substrate-free $P450_{cam}$ than for substrate-bound $P450_{cam}$. The larger λ would be due to the change in co-ordination that takes place in substrate-free $P450_{cam}$.

Despite being thermodynamically favourable in the presence of dioxygen the slower rate of the first electron transfer to substrate-free $P450_{cam}$ relative to substrate-bound P450 can be explained using Marcus theory. Electron transfer to substrate-free $P450_{cam}$ has a more positive free energy but, more importantly, is expected to have a much larger reorganisation energy which is the dominant factor affecting the kinetics of the electron transfer in both cases.

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