

Dynamics of Electron Transport in Cytochrome P450 Systems Studied at Sub-Zero Temperature*

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Experimentation in fluid mixed solvents (1:1 v/v phosphate buffer ethylene glycol) at sub-zero temperatures has permitted us to record the two univalent reductions of the bacterial cytochrome P450 by the natural electron donor putidaredoxin, without recycling or alternative pathway reactions. Dynamic evidence shows the formation of putidaredoxincytochrome complexes prior to electron transfer. The complex formation is rate limiting in the first reduction and in our experimental conditions. The kinetics of binding between the two oxidized proteins has also been recorded in the same medium under various conditions of concentration, temperature and ionic strength. At very low ionic strength, the rate is limited by electrostatic repulsion between the two negatively charge proteins; above $I = 0.03$ this effect appears negligible and the affinity seems to be governed by hydrophobic interaction free energy.

INTRODUCTION

The mechanism of camphor hydroxylation by the bacterial monooxygenase system has been resolved to show four main steps in the sequence of cytochrome P450 reactions^{1,2}. These are the binding of the substrate camphor (S) by the free ferric cytochrome (m^0) ($m^0 + S \rightleftharpoons m^{0s}$), reduction of the enzyme-substrate complex (m^{0s}) ($m^{0s} + e^- \rightarrow m^{rs}$), binding of molecular oxygen to give a ternary oxyferrous compound (m_{02}^{rs}) ($m^{rs} + O_2 \rightleftharpoons m_{02}^{rs}$), and the uptake of a second electron leading to the hydroxylated substrate SOH and the restoration of the free enzyme m^0 . Each step should now be analyzed in terms of the dynamic sequence of more elementary processes. Studies at sub-zero temperature in fluid mixed solvents are particularly well adapted to this problem. It allows the temporal resolution of reactions into elementary steps according to activation energies³, the stabilization of reactive intermediates, and the discrimination among individual pathways within a set of alternative reaction processes⁴.

This paper presents the application of this procedure to the dynamics of the first and second electron transfer from reduced putidaredoxin Pd⁻ to respectively m^{0s} and m_{02}^{rs} with special emphasis on the specific protein-protein interactions of the two enzymes.

EXPERIMENTAL

All the experiments are conducted in 1:1 (v/v) mixtures of phosphate buffer pH 7 and ethylene glycol (EGOH, Carlo Erba product). The protonic activity of this mixture

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is 7.5 at +20 °C and increases only slowly when the temperature decreases ($p_{a_H} = 7.55$ at -40 °C)^{8,9}. The buffer is 0.1 M except when specified; KCl and camphor concentrations are also specified in each case.

Spectra and kinetics are recorded with an Aminco-Chance DW2 spectrometer equipped for sub-zero temperatures⁷ or with a specially designed low temperature stopped-flow, already described⁸.

RESULTS

First electron transfer

The reduction of m^{os} by reduced putidaredoxin can be measured directly after the rapid mixing of these components in a stopped flow apparatus. Kinetics monitored by the absorbance decrease at 390 nm are shown in Figure 1 at different temperatures and Pd^- concentrations.

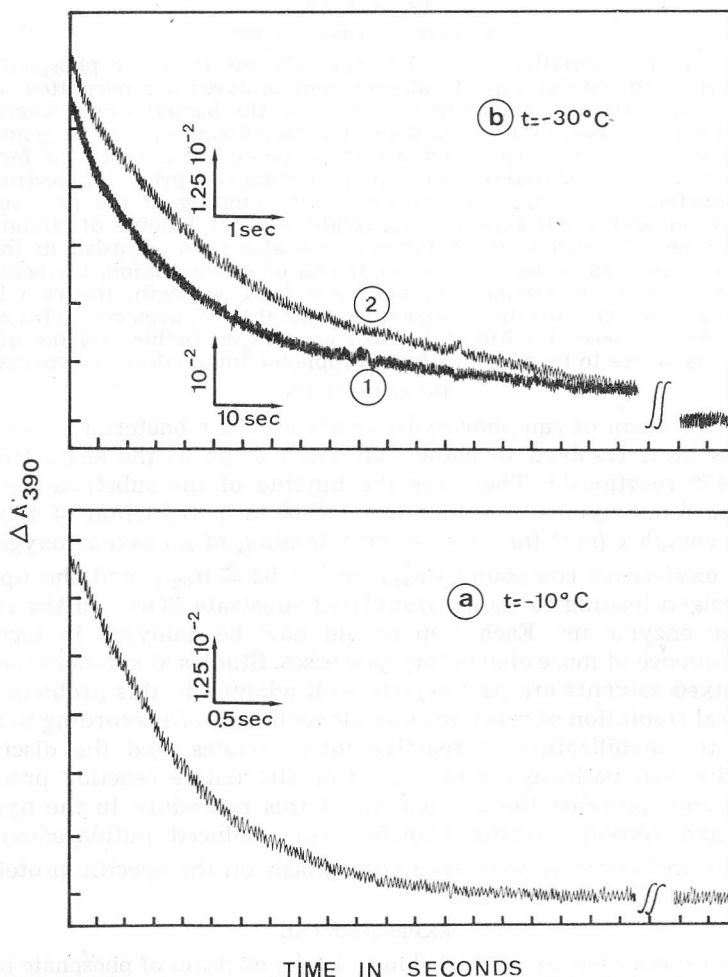


Figure 1. Kinetic traces of the reduction of m^{os} by Pd^- by stopped-flow at sub-zero temperatures. Solvent: 1 : 1 (v/v) mixture of 100 mM phosphate buffer pH 7 and EGOH, containing 200 mM KCl and 3 mM camphor as final concentrations. $e_o = [m^{os}] = 4.6 \mu M$. Monitoring wavelength: 390 nm. Figure 1a: $t = -10^\circ C$ $s_o = [Pd^-] = 19.7 \mu M$. Figure 1b: $t = -30^\circ C$ (1) $s_o = [Pd^-] = 3.8 \mu M$ $s_o = [Pd^-] = 25.3 \mu M$.

Under the conditions described above, control experiments show that putidaredoxin is still 100% reduced at the beginning of the reaction and that the direct reduction of either m^{OS} or Pd^0 by the excess dithionite present is too slow to be taken into account. Furthermore m_{02}^{rs} formation is avoided by the exhaustive deoxygenation of the solutions.

The extent of m^{OS} reduction (x_e), as measured from the total absorbance change during the reaction, is not 100% using initial stoichiometric concentrations of putidaredoxin (s_0) and cytochrome (e_0), but follows a smooth saturation curve when plotted as a function of s_0 , with a plateau at 60% reduction. Curves shown in Figure 2 for two temperatures indicate clearly that the reduction process is reversible.

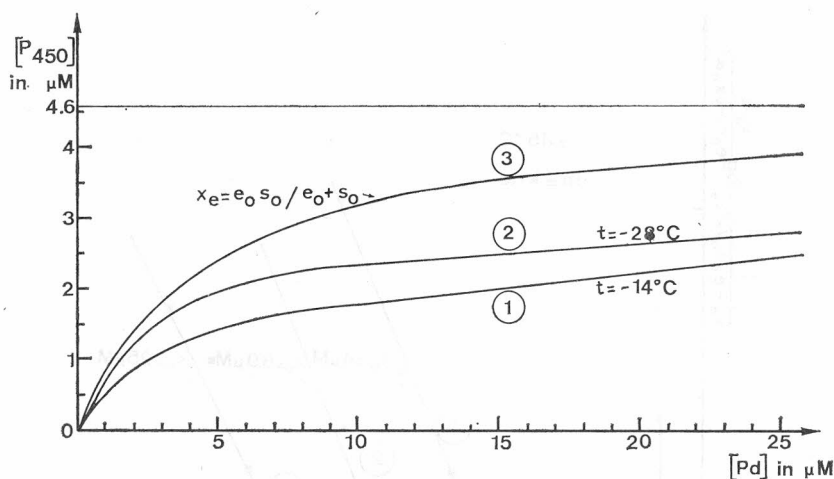
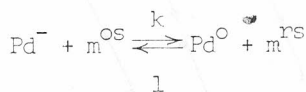


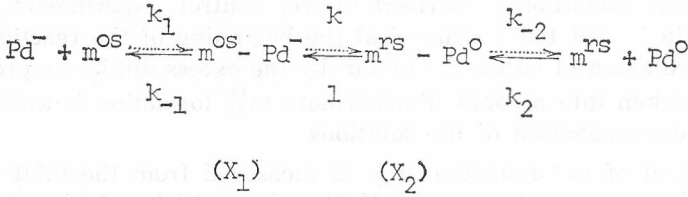
Figure 2. Final concentration of m^{rs} (x_e) as a function of the initial Pd^- concentration (s_0). Solvent as in Figure 1. (1) $t = -14^\circ C$ (2) $t = -27^\circ C$ (3) calculated curve obtained from the equation $x_e = e_0 s_0 / e_0 + s_0$

Also plotted in Figure 2 is the variation of x_e with increase in s_0 calculated according to $x_e = e_0 s_0 / e_0 + s_0$, which is the limiting expression for k approaching 1 of a simple reversible bi-molecular reduction process:



The theoretical values increase much more steeply than the experimental ones and reach as limit 100% reduction (curve 3, Figure 2). If $k \gg 1$ the increase of x_e would be much steeper, without a change in the limit value.

The difference between the experimental and theoretical plots clearly shows that the reduction is not a simple bimolecular reversible process and strongly suggests that the two proteins first interact to give a bimolecular complex of defined composition, in which the cytochrome is reduced by an intramolecular electron transfer from the putidaredoxin. Thus the following scheme may be proposed:



Bimolecular complexes between the two oxidized proteins have already been demonstrated by various techniques^{9,10}. More recently Gunsalus and Sligar measured a substantial increase of the redox potential of Pd upon binding

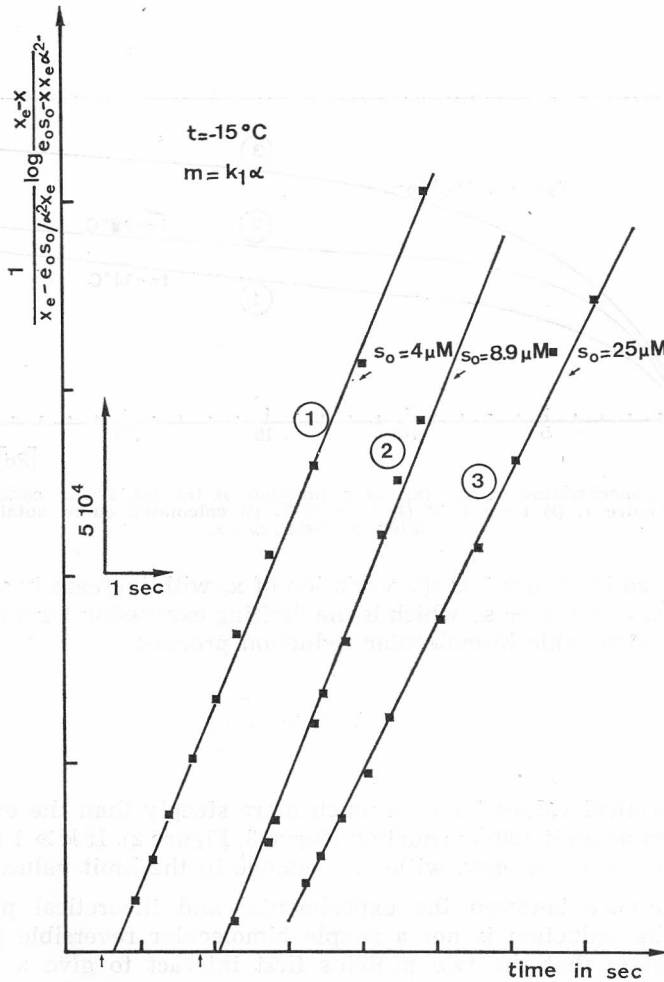


Figure 3. Plots of $\frac{1}{x_e - D} \log \frac{x_e - x}{D - x}$ as a function of time, with $D = e_0 s_0 / a^2 x_e$. Values of D , x_e and x are calculated from the experimental kinetic traces. $t = -14^\circ\text{C}$; $e_0 = 4.6 \mu\text{M}$ curve (1) $s_0 = 4 \mu\text{M}$ curve (2) $s_0 = 8.9 \mu\text{M}$ curve (3) $s_0 = 25 \mu\text{M}$.

with m^{os} (from -240 mV to -196 mV)¹¹. Such an increase reduces the free energy of electron transfer and is certainly the basis of the experimentally observed reversibility.

From the kinetic view point, one must determine whether the rate limiting step is the bimolecular complex (X_1) formation or electron transfer. Analysis of the experimental kinetics in terms of various mechanistic hypotheses provides the best fit by assuming that the rate limiting step is the binding of Pd^- to m^{os} , with a high affinity constant, followed by a fast electron transfer¹². Thus plots of

$$\frac{1}{e_o s_o / \alpha^2 x_e - x} \text{ Log } \frac{x_e - x}{e_o s_o / \alpha^2 x_e - x}$$

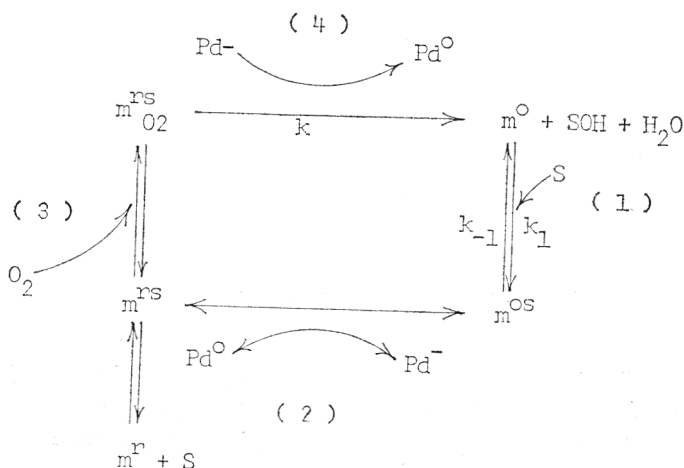
(where $\alpha = 1 + 1/k$) as a function of time should be linear with a slope $m = k_1 \alpha$ independent of s_o , as shown by Figure 3.

Assuming $1/k = 0.4$, (the value previously determined by Gunsalus *et al.*¹¹ under normal conditions of solvent and temperature), the association constant deduced from such plots is $k_1 = 3.5 \cdot 10^4 \text{ M}^{-1} \text{ sec}^{-1}$ at -15°C and the activation energy is $11 \pm 0.5 \text{ kcal/mol}$ ¹².

Note that at higher Pd^- concentration, $e_o s_o / \alpha^2 x_e$ is much higher than x and the second term of the logarithm varies only slightly with time. Thus a plot of $\log(x_e - x)$ as a function of time is linear, with a slope $m = k_1 e_o s_o / \alpha x_e$ practically proportional to s_o since x_e is almost stationary (see curves 1 and 2, Figure 2). Such linear Pd^- dependent semi-logarithmic plots are indeed observed experimentally¹².

Second electron transfer : the enzymatic decomposition of m_{02}^{rs} by Pd^-

The transfer of the second electron from Pd^- to m_{02}^{rs} is more difficult to study. This step of a closed cycle is irreversible, whereas all the other reactions are reversible:



Thus the decomposition of $m_{O_2}^{rs}$ is performed in the presence of an excess of the other components (S, O_2 , Pd^-) which have contributed to the ternary oxy-compound $m_{O_2}^{rs}$ formation and can recycle the free enzyme through reactions (1) (2) and (3). The speeds of these reactions are very fast at room temperature as seen when measured directly¹³ or extrapolated from the above low temperature measurements. Accordingly a two step procedure was used, which fully utilizes the cumulative effects of solvent and temperature on rate and equilibrium constants to allow a temporal resolution of this cycle.

Reaction (1) (addition of hydroxylable substrate) and (2) (first reduction) are performed at $+10^\circ C$. The concentration of free camphor is not saturating (20 to 30 μM while $K_d \approx 15 \mu M$; Marlène Molinié, personal communication) under these conditions of solvent and temperature but is still higher than the cytochrome concentration (1 to 3 μM). A continuous photochemical reduction of Pd^0 in the absence of oxygen shifts the equilibria (1) and (2) progressively towards the full reduction of both components, as shown by the optical spectra; in the final state the two proteins are in a stable association equilibrium:



The system is activated at a lower temperature ($-10^\circ C$ to $-40^\circ C$) by bubbling for 5–10 s with oxygen, which quickly binds (1 to 2 s at $-40^\circ C$) to the free or Pd^- -complexed m^{rs} (reaction 3). The much slower decomposition of $m_{O_2}^{rs} - Pd^-$ is then monitored by the appearance of m^0 (increase of absorbance at 406 nm which is isosbestic between m^0 and m^{os}). Figure 4 shows kinetic

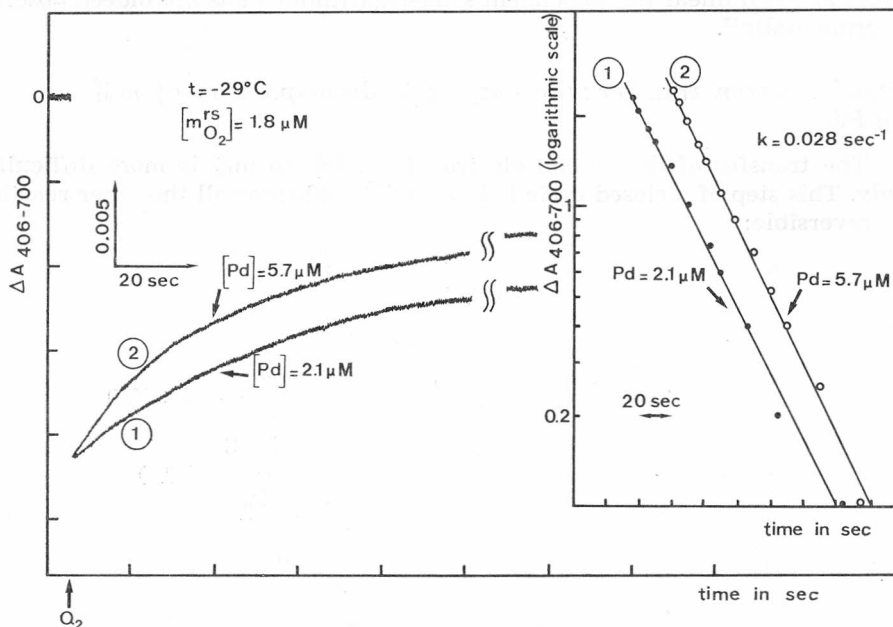
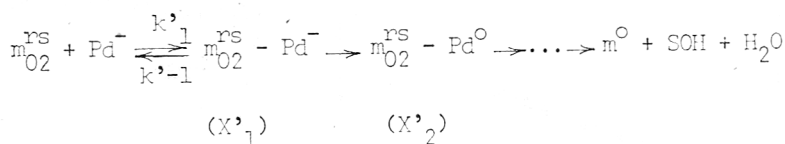


Figure 4. Kinetics of $m_{O_2}^{rs}$ decomposition by Pd^- at $-29^\circ C$. Solvent 1 : 1 v/v mixture of 0.1 M phosphate buffer pH 7 and ethylene glycol containing 30 μM camphor and 0.2 M KCl as final concentrations. Cytochrome 1.8 μM . $[Pd^-] = 2.1 \mu M$ (1) or 5.7 μM (2). Photochemical reduction followed by bubbling of oxygen (10 s). Absorption increase followed at 406 nm. The absorbance before oxygen addition is also indicated. Insert shows semi-logarithmic plots of $\Delta A_{406-700}$ (arbitrary units) versus time.

traces obtained at different Pd^- concentrations at $t = -29^\circ\text{C}$. Under these experimental conditions the decomposition is perfectly first order (insert of Figure 4) and monophasic, the rate constant depending only on the temperature and *not* on the initial Pd^- concentration. $k = 0.17 \text{ sec}^{-1}$ at -10°C and 0.011 sec^{-1} at -40°C . The measured activation energy is $11.5 \pm 0.5 \text{ kcal/mol}$.

One should point out that at such a low concentration, substrate combines only very slowly with the free enzyme (R. Lange *et al.* in preparation)¹³; furthermore Pd^- does not reduce m^0 because of its too low redox potential, the possibility of recycling m^0 through reaction (1) (2) and (3) is negligible and the recorded kinetics represent only *one turnover*, schematized by the following sequence:



Finally the yield of decomposition is not stoichiometrically related to the cytochrome nor putidaredoxin concentration, but follows a saturation curve with a plateau at 100% transformation and an affinity constant of approximately $1.5 \mu\text{M}$ for the electron donating species. At a higher temperature or camphor concentration, the kinetics are followed by a slow »drift« in the absorbance which represents the progressive decomposition of recycled $m_{\text{O}_2}^{\text{rs}}$.

Binding of Pd^0 to m^{os}

These reactions demonstrate the importance of protein association in the electron transfer processes from Pd^- to cytochrome P450. As already mentioned, such an association also occurs between the two oxidized proteins and may be followed by a Pd^0 induced shift towards the low spin of the high spin (HS) \leftrightarrow low spin (LS) equilibrium of m^{os} . The amplitude of this spin state transition is much greater at low temperatures, and the rate much slower thus facilitating the measurements of binding kinetics.

Figure 5 shows examples of the kinetics of HS to LS change after addition of Pd^0 at different ionic strengths and temperatures.

The rate and amplitude of the spin state change depend on many factors such as Pd^0 concentration, temperature, and ionic strength. Although a complete analysis is rather difficult, some characteristic features of this reaction must be pointed out. At very low ionic strength ($I = 0.015$) the reaction is extremely slow and monophasic first order, with a rate constant depending on the Pd^0 concentration (curve 1, Figure 5a). With increased I , the kinetics change progressively from monophasic to biphasic and become much faster (curve 2, Figure 5a), with a rate constant independent of the ionic strength (compare curves 1 and 2 Figure 5b). In the meantime the amplitude of the Pd^0 induced spectral change increases to a maximum at $I \approx 0.03$ and then decreases continuously (compare the amplitudes in the various curves).

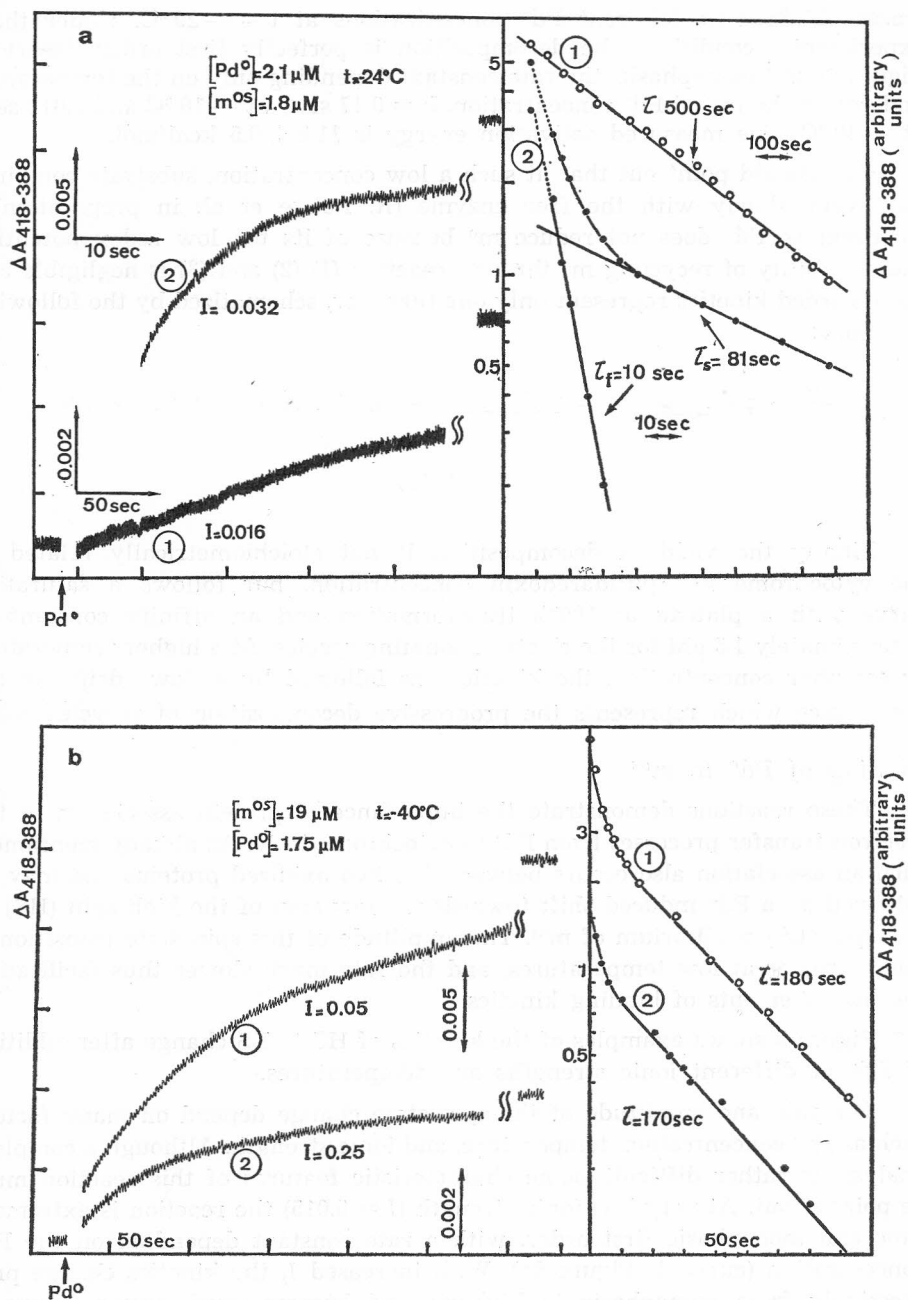


Figure 5. Kinetics of the HS \rightleftharpoons LS spin state change of m^{os} upon binding of Pd^0 at various temperatures and ionic strengths. Absorption increase followed at 418–388 nm. Solvent: 1:1 (v/v) phosphate buffer pH 7 – ethylene glycol containing 500 μ M camphor. Insert shows the semi-logarithmic plots of the corresponding kinetics. a) $t = -24^\circ C$. Final ionic strength of the mixed solvent $I = 0.016$; curve 1) without KCl added; curve 2) with 0.016 M KCl. b) $t = -40^\circ C$. Final ionic strength of the mixed solvent $I = 0.05$; curve 1) without KCl; curve 2) with 0.2 M KCl.

It could be argued that the variations in the amplitude of the $\text{HS} \rightleftharpoons \text{LS}$ change reflect an ionic strength dependence of the affinity of Pd^0 towards m^{os} . To solve this point, Lineweaver-Burk plots of $1/\Delta A$ versus $1/[\text{Pd}^0]$ were constructed at different ionic strengths (Figure 6). Rather large errors occur in the values plotted due to the small amplitude of the optical changes when $[m^{\text{os}}]$ is low compared to $[\text{Pd}^0]$. Within the experimental errors, the dissociation constant K_d is found to be largely independent of I in the two regions described. Thus the presence of KCl does not affect the tightness of the binding but does directly affect the HS/LS equilibrium constant of the $m^{\text{os}}-\text{Pd}^0$ complex, as already observed for the Pd^0 -free cytochrome m^{os} (unpublished results). The main values deduced from such plots are at -34°C $K_d = 40 \pm 10 \mu\text{M}$ and at -25°C $30 \pm 6 \mu\text{M}$.

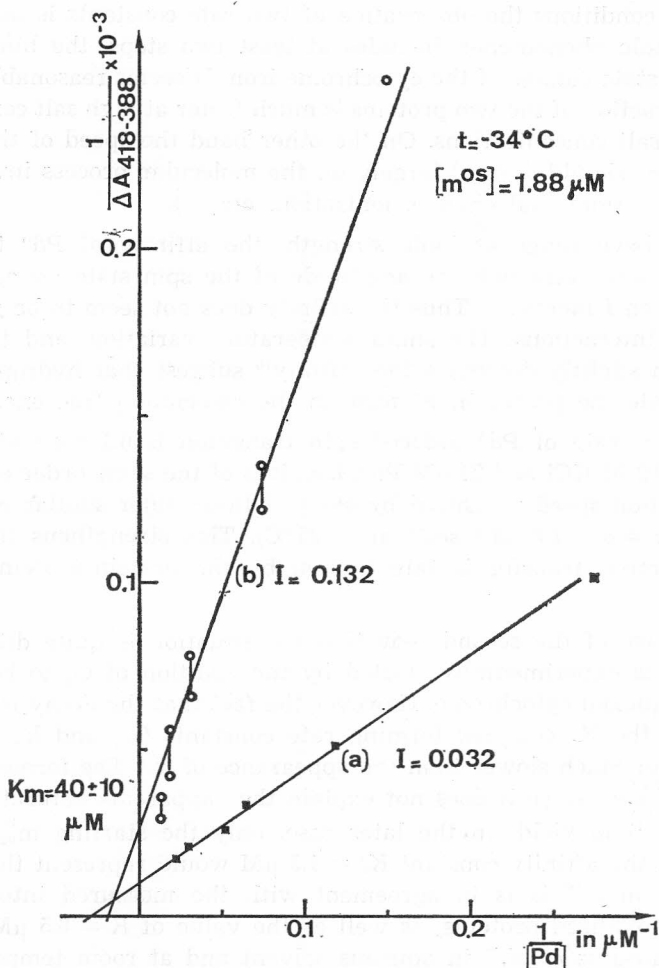


Figure 6. Reciprocal plot of the absorption increase between 418 nm and 388 nm (ΔA) as a function of Pd^0 concentration. $t = -34^\circ\text{C}$. Solvent: 1 : 1 (v/v) phosphate buffer pH 7 — ethylene glycol containing 1.2 mM camphor and of final ionic strength $I = 0.032$. m^{os} concentration: $1.88 \mu\text{M}$. (a) no KCl added (b) 0.100 M KCl added.

DISCUSSION

The preceding results show that the dynamics, *i. e.* formation rate and stability, of the P450-Pd interaction, play an important role in the two univalent reductions of the cytochrome P450 cycle.

As both proteins are very acidic (isoionic point of 3.4 and 4.55 for respectively Pd⁰ and m^{0s}) their close interaction should theoretically be hidden by repulsive electrostatic forces. The results show that the association rate constant in the ferric state depends on at least two types of forces. The extremely slow interaction at very low ionic strength is most probably rate-limited by electrostatic repulsion between the two negatively charged proteins whereas at higher ionic strength, the rate is much faster and independent of ionic strength. Thus another process becomes rate limiting in the Pd⁰ induced HS ↔ LS transition. Under these conditions the observation of two rate constants is not surprising since the whole phenomenon includes at least two steps, the binding of Pd⁰ and the spin state change of the cytochrome iron. It seems reasonable to believe that the interaction of the two proteins is much faster at high salt concentrations than at low salt concentrations. On the other hand the speed of the electronic rearrangement should depend largely on the molecular process involved (local or global conformational change, ionization, *etc.*...).

In the above range of ionic strength, the affinity of Pd⁰ towards m^{0s} remains the same, although the amplitude of the spin state change decreases markedly when *I* increases. Thus the affinity does not seem to be governed by electrostatic interactions. The small temperature variation, and the effect of EGOH which slightly decreases the affinity¹⁰ suggest that hydrophobic interactions provide the predominant term in the association free energy.

The faster rate of Pd⁰ induced spin transition is 0.1 sec⁻¹ at -25 °C in presence of 0.2 M KCl and 21 μM Pd⁰, *i. e.*, it is of the same order of magnitude as the reduction speed measured by stopped flow under similar experimental conditions ($k = 8 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ at -25 °C). This strengthens the idea that the first electron transfer is rate limited by the protein-protein interaction speed.

In the case of the second reduction the situation is quite different since the reaction is experimentally started by the addition of O₂ to both the free and Pd⁻-complexed cytochrome. However the fact that the decay is monophasic implies that the X₁ complex forming rate constants (k'_1 and k'_{-1}) are either much faster or much slower than the appearance of m⁰. The former hypothesis is rather unlikely since it does not explain the »apparent« saturation curve of the decomposition yield. In the later case, only the starting m^{rs}₀₂ would decompose and the affinity constant $K_d = 1.5 \text{ μM}$ would represent the affinity of Pd⁻ towards m^{rs}. This is in agreement with the measured interaction rate between the oxidized proteins, as well as the value of $K = 0.5 \text{ μM}$ previously found by Gunsalus *et al.*¹¹ in aqueous solvent and at room temperature. The large difference between this affinity constant and the measured affinity of Pd⁰ towards m^{0s} ($K_d \approx 40 \text{ μM}$) raises the question of the probable existence of two types of binding between the two proteins as already suggested¹⁰.

The present results do not give clear indications concerning the stoichiometry of the various dienzyme complexes involved in the binding of Pd⁰ (Pd⁻) to m^{0s} (m₀₂^{rs}) and this point is in need of further study.

Note also that many intermediate compounds must exist between X'₂ and the free ferric cytochrome, which remain up to now spectrophotometrically undetectable but are under close examination.

In conclusion, the present low temperature experimentation of the bacterial methylene hydroxylase allowed a partial temporal resolution of the two electron transfers from putidaredoxin to cytochrome. This was made possible essentially through the differential effect of solvent and temperature on rate constants. The main interest of such studies is to give access not only to the enzyme-substrate interaction, but also to the dynamics of protein-protein interaction, which, through the sequential formation and dissociation of functional multi-enzyme complexes, is the basis of reactivity and control of multi-component functions. This procedure could be applied to many other soluble or membraneous multi-component systems.

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DISCUSSION

K. Ruckpaul:

In which way do the kinetics you have measured depend on the non-aqueous solvent? Do you find differences in the kinetics behaviour in dependence to the non-aqueous solvent used?

P. Debey:

I do not know since we have used only ethylene glycol as organic solvent. The values of the rate constants obtained by extrapolation of our values to higher temperatures (above 0 °C) agree quite well with previous results of Gunsalus *et al.*, but they show some discrepancy in regard to the more recent results of Tom Pederson from Dr. Gunsalus's laboratory. These discrepancies could be interpreted as a solvent effect on the affinity of Pd⁺ towards m^{os}. We are now considering this point more carefully.

H. Schleyer:

Are there »breaks« in the time course for Pd⁺ + m^{so} at higher than 1 : 1 molar ratio, while monophasic reaction is obtained at 1 : 1 ratio?

P. Debey:

There are no »breaks« in the kinetics whatever the ratio of the two proteins.

M. J. Coon:

Do the putidaredoxin — cytochrome complexes in the various reduction states have spectra different from those of the parent compounds? If so, does that affect the interpretation of your kinetic measurements at various wavelengths?

P. Debey:

The putidaredoxin — oxydized cytochrome is more low-spin than the free cytochrome. For the reduced putida — reduced cytochrome there is an almost undetectable change. For the mixed complexes (reduced putidaredoxin — oxydized cytochrome, X₁, or oxydized putidaredoxin — reduced cytochrome, X₂) we do not know and I agree with you that one must be careful about the interpretation of the kinetics. Furthermore, the amplitude of these changes (at least for the Pd^o — m^{os} complex) is much lower than the spectral change due to reduction of m^{os} under our conditions of high ionic strength (200 mM KCl) and we have made sure that the kinetics are the same at different wavelengths.

I. Blanck:

The high-spin/low-spin transition is biphasic in your experiments, and the time is in the range seconds. The spin equilibrium interchange of metmyoglobin is in the microsecond range. What about the somewhat slower reaction mechanism in the cytochrome system — it cannot be only a temperature difference?

P. Debey:

I agree with you. There are in my opinion two interpretations of these rather slow kinetics. Firstly, we know that in metmyoglobin (or haemoglobin) there is a water molecule as ligand. In the case of cytochrome P450 nobody knows and if the displacement of another ligand of the protein is involved in this spin state transition, one could expect it to be rather slower than in the case of myoglobin. Secondly, it could be that we have here a rate limiting conformational change of the cytochrome upon binding of Pd^o, which then induces the spin state change. Since a conformational change is involved one could also expect it to be a rather slow process.

H. Rein:

Do you mean that there is a spin equilibrium in P450?

P. Debey:

Yes. In the camphor-free and camphor-bound P450 a temperature dependent spin equilibrium exists. It has been shown by Dr Sligar in Dr Gunsalus' laboratory and also by us. It seems that by adding Pd^o you simply disturb this spin equilibrium.

SAŽETAK

Dinamika prijenosa elektrona u sistemima citokroma P450 ispod 0 °C

*P. Debey, G. Hui Bon Hoa i I. C. Gunsalus**

Eksperimentima u tekućim miješanim otapalima (1 : 1 v/v fosfatni pufer — etilen glikol) na temperaturama ispod 0 °C registrirana su dva jednovalentna stupnja redukcije bakterijskog citokroma P450 prirodnim donatorom elektrona, putidaredoksinom, bez recikliranja ili alternativnih postranih reakcija. Pokazalo se da kompleks putidaredoksina s citokromom nastaje prije prijenosa elektrona. Stvaranje tog kompleksa određuje ukupnu brzinu redukcije, dok je sam prijenos elektrona, unutar kompleksa, brz. Kinetika vezivanja obaju oksidiranih proteina praćena je uz različite uvjete (koncentracija, temperatura i ionska jakost). Kod vrlo malih ionskih jakosti brzina interakcije dvaju negativno nabijenih proteina ograničena je njihovim elektrostat- skim odbijanjem, a kod većih je taj efekt zanemariv i afinitet je čini se određen slo- bodnom energijom hidrofobnih interakcija.

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