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# *AN OPTICAL BIOSENSOR STUDY OF THE INTERACTION PARAMETERS AND ROLE OF HYDROPHOBIC TAILS OF CYTOCHROME P450 2B4, $b_5$ AND NADPH-FLAVOPROTEIN IN COMPLEX FORMATION.*

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SUMMARY

The real-time interactions of membrane proteins - cytochrome P450 2B4, NADPH cytochrome P450 reductase and cytochrome $b_5$ - were studied by use of an optical biosensor system. The association and dissociation rate constants for the individual complexes were measured and the affinities of the redox partners for each other were estimated. The association rate constants of these complexes were found to be close to the diffusion limit and their dissociation rate constants were in the order of $1s^{-1}$. A dominant role of the interaction of the membraneous hydrophobic fragments in the formation of productive electron transferring complexes between the proteins was demonstrated.

***Keywords***: *cytochrome P450 2B4, NADPH cytochrome P450 reductase, cytochrome $b_5$, kinetic constants, productive complexes, optical biosensor method.*

## INTRODUCTION

The importance of studies on cytochrome P450 2B4-containing monooxygenase systems is obvious from their ability to oxidize drugs, toxins, carcinogens, mutagens and other xenobiotics. Estimation of the kinetic and thermodynamic parameters for redox partners interaction - particularly of rate constants for complex formation and decay - and their comparison with rate constants for electron transfer and hydroxylation are essential for a better understanding of mechanisms governing such systems. Complex formation involves the appearance of both productive and nonproductive adducts of redox partners with intermolecular electron transport either occurring or not occurring, respectively. In the present study, the kinetic constants for protein-protein interaction were determined by the optical biosensor method based on the principle of frustrated total internal reflection. This method is direct, i.e. it permits immediate

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real - time measurement of the total amount of complexes formed. Importantly, comparison of the kinetic and thermodynamic parameters thus obtained with the electron transfer rate constants in these complexes enabled us to estimate the proportion of productive complexes within the overall number of adducts.

Full-length enzymes - cytochrome $b_5$ (d-$b_5$), NADPH cytochrome P450 reductase (d-Fp), and cytochrome P450 2B4 (d-2B4) - are amphipatic proteins containing hydrophobic membrane fragments capable of being incorporated into the lipid bilayer. These fragments may be removed by tryptic digestion to generate t-$b_5$ or t-Fp or by gene expression - to produce t-2B4 [1,2]. The present paper describes a comparative optical biosensor study of complex formation parameters for the full-length and truncated forms of 2B4, $b_5$ and Fp. Based on these data, the dominant role of hydrophobic membrane tails in productive complex formation is demonstrated.

## MATERIALS AND METHODS

Phenobarbital-induced rabbit liver microsomal d-2B4 and d-FP were prepared and quantified as described earlier [3]. Purified tryptic fragments of t-$b_5$ and t-FP were obtained from rabbit liver microsomes [1]. Truncated cytochrome P450 2B4 (t-2B4) lacking the N-terminal residues 2-27 was expressed in E-.coli and purified to apparent homogeneity as described elsewhere [2].

Monomerization of isolated d-Fp and d-2B4 was carried out as described previously [3] except that in some cases 100 mM K-phosphate buffer was replaced by 500 mM buffer solution. Monomerization of isolated d-$b_5$ was carried out in 500 mM K- phosphate buffer by the same procedure.

Measuring of the association ($k_{on}$) and dissociation ($k_{off}$) rate constants for the complexes between the redox partners was carried out on the "resonant mirror" optical biosensor IAsys (FISONS, UK) [4-6]. The immobilization of d-2B4, d-Fp and d-$b_5$ on the carboxymethylated matrix of the optical biosensor was carried out in 10 mM sodium acetate buffer, pH 4.0. The immobilization of t-2B4 was carried out in 5 mM maleate buffer, pH 6.0.

The binding of immobilized proteins to their respective monomeric redox partners was studied by adding protein aliquots in 500 mM K-phosphate buffer containing 0.25 g/l Emulgen 913. Every protein aliquot was added in large excess in comparison with the immobilized ligate after buffer washing. The association rate constants were determined under pseudo-first-order reaction conditions. Data fitting was performed using the FASTfit program (FISONS) based on the equations: $R = R_0 + R_1 * (1 - \exp(-f*t))$, where R is a response from device, t = time period, $R_0$ = response at t=0; $R_1$- response at $t=\infty$ minus $R_0$, $f = k_{on} * c + k_{off}$, and c is ligand concentration [6]. On enzyme immobilization, some of its amino groups participating in redox partners' complex formation may be also involved in the immobilization process. To minimize the error, the kinetic constants were measured upon immobilization of each partner in a given pair. Kinetics of NADPH - dependent reduction of $b_5$ and 2B4 in the presence of benzphetamine was registered under anaerobic conditions at $\Delta$ 424 - 408 nm and $\Delta$ 450 - 490 nm, respectively. In the presence of $b_5$, the second-electron-transfer rate constant was calculated from the difference of the rate constants for NADPH oxidation and superoxide anion formation during 1-electron reduction of $O_2$ on 2B4.The NADPH oxidation and superoxide anion generation rate constants were measured as described by Zhukov and Archakov [7].

RESULTS AND DISCUSSION

In this study, d- and t- 2B4 as well as d-Fp and d- $b_5$ were immobilized on the surface of the measuring cuvette, allowing us to determine the parameters of complex formation between immobilized proteins and their respective redox partners. Figure 1 presents the d-2B4 immobilization graph; the curves for the other enzymes were analogous and hence are not shown here. The surface concentrations of immobilized (im) enzymes for d-$2B4_{im}$, t-$2B4_{im}$ d-$Fp_{im}$, d-$b_{5im}$ were measured and found to be $7.4*10^{-14}$ mol/mm$^2$, $5.0*10^{-14}$ mol/mm$^2$, $3.8*10^{-13}$ mol/mm$^2$ and $4*10^{-14}$ mol/mm$^2$, respectively. The binding curves for the pair d-$2B4_{im}$/d-$b_5$ are presented in Figure 2. The curves for all the other pairs have analogous characteristic profiles and are not shown either. One can see both the increase of the signal, providing evidence for the binding to occur after the addition of d-$b_5$ into the cuvette, and the attenuation of the signal, evidencing dissociation of the complex after buffer addition. Enhancement of the ligand concentration results in amplification of the signal. The $k_{on}$ and $k_{off}$ for the redox pairs studied, as calculated from these sensorgrams, do not depend on whichever partner is immobilized on the dextran matrix (Fig.3). Therefore Table 1 presents only the mean kinetic constants values for the redox partners under investigation. Included for comparison are also the electron transfer rate constants for the protein pairs studied. It can be seen that the $k_{on}$ values for the membrane - fragments - containing pairs are in the order of $10^6$ $M^{-1}$ $s^{-1}$, suggesting that complex formation rate may be limited by diffusion [8]. The dissociation rate constant for the pair d-$b_5$/d-Fp is 0.5 $s^{-1}$ and the

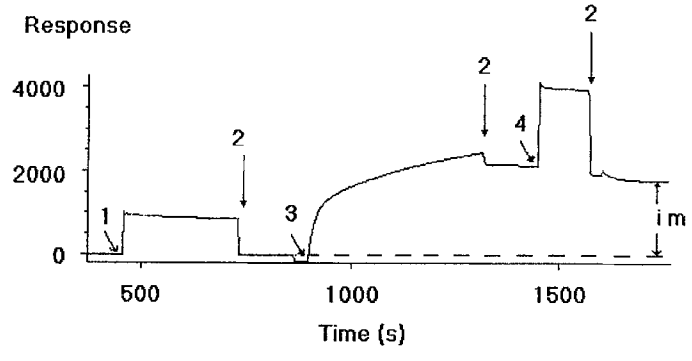


Figure 1. The immobilization curve of d-cytochrome P450 2B4 attachment to the carboxymethylated dextran matrix of optical biosensor cell, activation with EDC&NHS (1), buffer wash (2), addition of 20 μl of 5 μM 2B4 in 500 mM K-phosphate buffer (3), deactivation with 1 mM ethanolamine (4), amount of immobilized protein (im), T=25C°.

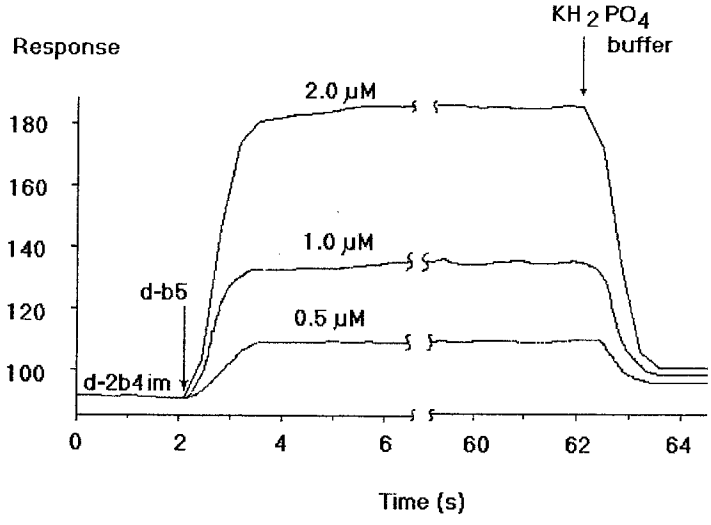


Figure 2. The binding of d-cytochrome $b_5$ monomers at various concentrations to the immobilized d-cytochrome P450 2B4. The incubation mixture contained 500 mM K-phosphate buffer with 0.25 g/l Emulgen 913, pH 7.4. T= 25 C°. The arrows indicate the d-$b_5$ aliquot and buffer addition time.

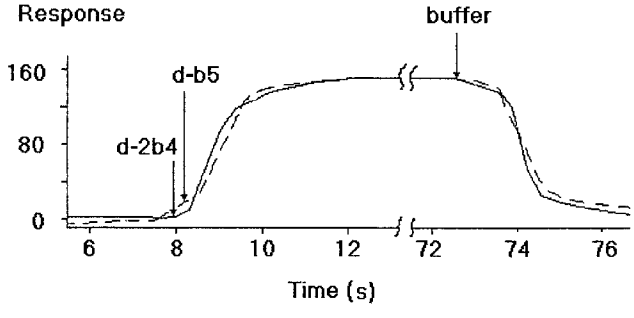


Figure 3. The binding of d- cytochrome P450 2B4 monomers to immobilized d- cytochrome $b_5$ (___) and the binding of d- cytochrome $b_5$ monomers to immobilized d-cytochrome P450 2B4 (- - -). The incubation mixture contained 500 mM K-phosphate buffer with 0.25 g/l Emulgen 913, pH 7.4. T= 25 C°. The arrows indicate the d-2B4 and d-$b_5$ aliquot and buffer addition time.

Table 1.
Parameters of the redox partners measured by the optical biosensor methods

| Pairs | $k_{on}$*$10^6$, $M^{-1}$ $s^{-1}$ | $k_{off}$, $s^{-1}$ | $K_d$, μM | τ, s | $k_e$, $s^{-1}$ |
|---|---|---|---|---|---|
| d-2B4+d-Fp | 2.0±0.6 | 0.7±0.2 | 0.3±0.1 | 1.4±0.4 | 1.8±0.5 |
| d-2B4+t-Fp | 0.10±0.05 | 1.3±0.1 | 13±9 | 0.8±0.1 | 0 |
| d-2B4+d-$b_5$ | 2.5±0.2 | 0.5±0.2 | 0.2±0.1 | 2.0±0.8 | 1.00±0.03 |
| d-2B4+t-$b_5$ | 0 | | | | 0 |
| d-$b_5$+d-Fp | 1.6±0.9 | 0.5±0.2 | 0.3±0.2 | 2.0±0.8 | 0.4±0.1 |
| t $b_5$+d-Fp | 0 | | | | 0.4±0.1 |
| t-2B4+d-Fp | 0 | | | | 0.02±0.01 |
| t-2B4+t-Fp | 0 | | | | n.d. |
| t-2B4+d-$b_5$ | 0 | | | | n.d. |
| t-2B4+t-$b_5$ | 0 | | | | n.d. |

electron transfer rate ($k_e$) is 0.4 $s^{-1}$. Thus the characteristic life span of the complex ($\tau=1/k_{off}$) is 2.0 s, while the electron transfer time ($\tau_e=1/k_e$)= 2.5 s. From these findings it follows that the probability of charge transfer ($P=\tau/\tau_e$) from the d-Fp molecule to d-$b_5$ during the existence of the complex has a value of 0.8. Therefore the proportion of productive complexes in this case amounts to 80% of the total. For the pairs d-2B4/d-$b_5$ and d-2B4/d-Fp the rates of protein-protein electron transfer are, respectively, twice and 2.6 times higher than the dissociation rate of their complexes; hence, for each complex while it exists the probability of electron transfer from the $b_5$ and d-Fp molecules to 2B4 is close to 1 (P cannot exceed 1 by definition). Thus it would seem that all the complexes formed are productive and during the life span of each of these complexes 2 electrons can be transferred.

To elucidate the role of membrane fragments of the redox partners in productive complex formation, we have undertaken to study the interaction of t-$b_5$, t-Fp and t-2B4 with their full-length redox partners. The results are presented in Table 1 along with the data on occurrence or non-occurrence of electron transfer in these complexes. Clearly, no complex formation of t-$b_5$ with d-Fp is observed although the electron transfer does occur. This can be interpreted as indicating that the intermolecular electron transfer for this pair is effected through random collisions. Another situation is given by the redox pair d-$b_5$/d-Fp, where the complex formation is accompanied by charge transfer. Formation of this productive complex as well as the lack of its production with the pair t-$b_5$/d-Fp show that the hydrophobic tail of $b_5$ is essential for complex formation. The lack of complexes for the pairs t-$b_5$/d-2B4 and d-$b_5$/t-2B4, along with formation

of the complex by the pair d-$b_5$/d-2B4, are likewise indicative of the hydrophobic tail requirement for the d-$b_5$/d-2B4 productive complex formation. Complex formation was also observed in the case of the pair Fp/2B4 - with both d -and t- Fp. Affinity of t-FP and d-2B4 toward each other was about 1.5 order of magnitude lower than for the d-Fp/d-2B4 couple, with all t-Fp/d-2B4 complexes being nonproductive.. No complexing of d- or t-Fp with t-2B4 was observed. This again points to the essential role of the hydrophobic tails of d-2B4 and d-Fp in their productive complex formation. For t-2B4 and d-Fp the electron transfer reaction is realized by random collision but with a lower rate than in productive complexes.

Thus the intermolecular electron transfer in protein pairs studied may occur both on random collisions and during complex formation ; the availability of membrane fragments is an essential requirement for productive complex formation to occur. For the full-length protein pairs d-2B4/d-$b_5$, d-2B4/d-Fp, d-$b_5$/d-Fp the percent of productive complexes is on the order of 100%. In the case of d-2B4/d-$b_5$ and d-2B4/d-Fp the transfer of two electrons is possible for each pair.

It is to note that current literature [9] reports kinetic data for some protein pairs as determined by the fluorescence quench assay ($k_{on}$ d-Fp/d-2B4 = $6.5*10^5 M^{-1} s^{-1}$ , $k_{off}$ = $0.025\ s^{-1}$ ). Our data compare favorably with the $k_{on}$ value cited above. However, the discrepancy by one order of magnitude regarding the $k_{off}$ constants is possibly due to the use of different buffer systems as well as to the inability of the fluorescence quenching method to register those complexes in which no quenching is observed.

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