

Blue Light Mediated Photoreduction of the Flavoprotein NADPH-Cytochrome P450 Reductase. A Förster-Type Energy Transfer

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The absorption spectra and the corresponding molar absorption coefficients of the fluorophores umbelliferone, FAD and FMN and of the FAD and FMN containing flavoprotein NADPH-cytochrome P450 reductase of different oxydation-reduction states are documented.

Binding spectra of the ligand umbelliferone with the CYP2B1:NADPH-cytochrome P450 reductase-complex were determined by difference spectroscopy. The Scatchard plot of the equilibrium ligand binding shows a high affinity part and a low affinity part of 12 and 34 umbelliferone binding sites per CYP2B1:reductase-complex molecule, respectively.

The fluorescence excitation and emission spectra of the donor molecule umbelliferone and the acceptor molecules FAD and FMN are given. The fluorescence spectra of the reaction components under test conditions of CYP2B1-dependent 7-ethoxycoumarin-O-deethylase are measured. The excitation energy transfer from the donor umbelliferone ($\lambda_E=380$ nm; $\lambda_F=460$ nm) to the acceptor molecule FMN ($\lambda_E=465$ nm; $\lambda_F=525$ nm) was examined under assay conditions. The results demonstrate that a radiationless Förster-type energy transfer takes place in the presence of the CYP2B1:reductase-complex. It turned out that this effect is a function of the protein complex-concentration.

The data presented here combined with previously made observations by Müller-Enoch (Müller-Enoch D. (1994), Z. Naturforsch. **49c**, 763–771) support the finding that the umbelliferone molecules, $n = 12-34$, bound per mole of CYP2B1:reductase-complex, transfer their absorbed light energy radiationless to the FAD binding domain. The complex formed containing 12 or 34 molecules of umbelliferone provides absorption coefficient values at $\lambda = 380$ nm of 78 and 221 $\text{mm}^{-1} \cdot \text{cm}^{-1}$, respectively. The Förster-type energy transfer from the donor umbelliferone to the acceptor FAD not only leads to a light activation of the singlet state of FAD but also to a conformational change of the amino acids close to the FAD binding side to favour the encaging of the FAD* triplet state which reacts with the NADPH to form the FADH₂ reductase. Due to this process the overall reaction can start with the unquenched excited FAD* triplet state as an intermediate which is about 30 kJ/mol lower in energy than the dark reaction.

Introduction

The activation of the cytochrome P450 dependent monooxygenase system by blue light ($\lambda=420-440$ nm) was first published by Müller-Enoch and Gruler (1986), and Häberle *et al.* (1990). The light-induced enhancement of the enzyme activity can only be effective with the intermediate states which are rate-limiting in the catalytic cycle

(White and Coon, 1980) and when the enzyme system is able to absorb this blue light.

Using a reconstituted CYP2B1:NADPH-cytochrome P450 reductase system it was demonstrated, that the 7-ethoxycoumarin-O-deethylation or the scoparone-O-demethylation activities were increased by about one to three times on adding to the reaction mixtures their own fluorescent products, umbelliferone or scopoletin, and irradiating the reaction mixture $\lambda_E=365$ nm or $\lambda_E=398$ nm, respectively (Müller-Enoch, 1994).

It was argued that the fluorescent products function as photoreceptors and phototransducers, of the protein complex because they are in close proximity to the active center. In a previous paper we have shown that the absorbed light energy pro-

Abbreviations: CYP2B1, liver microsomal cytochrome P450; PB, phenobarbital; U, umbelliferone; FMN, flavin mononucleotide; FAD, flavin-adenine dinucleotide.

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vides a reaction path with a lower activation energy (Müller-Enoch, 1994).

In this study we will provide evidence demonstrating, how the photoacceptor umbelliferone is able to transfer its excitation energy, $\lambda_E=380$ nm, and its fluorescence energy, $\lambda_F=460$ nm, radiationless to the fluorophores FAD and FMN ($\lambda_E=465$ nm; $\lambda_F=525$ nm) of the NADPH-cytochrome P450 reductase to accelerate the rate limiting step in the over-all reaction. The energy transfer can be rationalized by the photoreduction of the 2-electron-containing reductase (FAD/FMNH₂) with NADPH to the 4-electron reduced form (FADH₂/FMNH₂).

Materials and Methods

Materials

7-Ethoxycoumarin was purchased from Aldrich-Chemie GmbH (Steinheim, Germany). 7-Hydroxycoumarin (umbelliferone) was purchased from EGA-Chemie KG (Steinheim, Germany) and recrystallized twice from hot water. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were purchased as sodium and disodium salts, respectively, from Sigma Chemie GmbH (Deisenhofen, Germany). All other chemicals and biochemicals used were of the highest purity available and obtained from E. Merck (Darmstadt, Germany) and Boehringer Mannheim GmbH (Mannheim, Germany). The materials used for the purification of CYP2B1 and the NADPH-cytochrome P450 reductase are described by Guengerich *et al.* (1982).

Animals and induction procedure

Male Sprague-Dawley rats, weighing between 250–300 g, were used. Treatment with phenobarbital (PB) consisted of the addition of 0.1% (w/v) PB to the drinking water for 6 days prior to sacrifice. The livers were removed and placed in ice-cold 0.25 M sucrose.

Purification of CYP2B1 and of NADPH-cytochrome P450 reductase

CYP2B1 (EC 1.14.14.1) and NADPH-cytochrome P450 reductase (EC 1.6.2.4) were purified to electrophoretic homogeneity out of microsomal fractions prepared from PB-treated rats as de-

scribed by Guengerich and Martin (1980). The SDS-PAGE pure CYP2B1 and the reductase had specific contents of 14.72 nmol/ml and 23.33 nmol/ml, respectively. The specific contents of the CYP2B1 and the reductase were determined by the methods of Omura and Sato (1964) and Yasukochi and Masters (1976), respectively. Protein was determined by the method of Lowry *et al.* (1951).

Reconstitution of the CYP2B1 with the NADPH-cytochrome P450 reductase

The purified CYP2B1 and the NADPH-cytochrome P450 reductase were mixed in a 1:2 molar ratio and diluted with 0.1 M Tris-(hydroxymethyl)-aminomethane-chloride buffer pH 7.6 (Tris/HCl buffer pH 7.6) to yield final concentrations of CYP2B1 and reductase of 1.64 and 3.28 μ M respectively. This enzyme mixture was allowed to stand for 1–1.5 h at room temperature as described by Müller-Enoch (1993). Aliquots of this reconstituted system (16 μ l=26.17 pmol CYP2B1 and 52.34 pmol reductase) were used for all experiments, unless otherwise stated.

Spectrophotometry

Absolute spectra and difference spectra of the CYP2B1 and the reductase were recorded from 370–700 nm using a Varian Cary spectrophotometer model 219 from Varian GmbH (Darmstadt, Germany). The same spectrophotometer was used to measure the absorption spectra of 10^{-6} to 10^{-5} M solutions (0.1 M Tris/HCl pH 7.6) of 7-ethoxycoumarin, umbelliferone, FMN, FAD and NADPH in the wavelength region of 270–550 nm against the buffer solution as a reference.

Difference spectra

The spectral interaction of the umbelliferone with the reconstituted CYP2B1:NADPH-cytochrome P450 reductase-complex in the presence of NADPH was studied by difference spectroscopy. A solution of CYP2B1 (0.48 μ M), reductase (0.58 μ M) and NADPH (0.125 mM) in a total volume of 800 μ l 0.1 M Tris/HCl buffer pH 7.6 was placed in the sample cuvette. The reference cuvette contained only NADPH (0.125 mM) in 800 μ l 0.1 M Tris/HCl buffer. After the base line had been

established, binding spectra were recorded after each addition of small volumes (few μl) of umbelliferone solutions (10^{-3} M in 0.1 M Tris/HCl buffer pH 7.6) in the concentration range of 2.5–30 μM to both cuvettes.

Fluorometry

The fluorescence activation and emission spectra of 7-ethoxycoumarin, umbelliferone, FMN, FAD, NADPH and the enzymes CYP2B1 and reductase were recorded with a Jobin Yvon spectrophotometer, model IY 3D Instruments S.A. (Unterhaching, Germany) in a quartz cuvette of 10 mm light path; Type 104 F-QS from Hellma (Freiburg, Germany). The optical band width was 4 nm. The uncorrected excitation and emission spectra of all compounds in 0.1 M Tris/HCl buffer pH 7.6, were recorded in the wavelength range from 310–600 nm, respectively.

CYP2B1-dependent 7-ethoxycoumarin-O-deethylase-activity

The 7-ethoxycoumarin-O-deethylase activity of the reconstituted CYP2B1:NADPH-P450 reductase system was assayed using the continuously fluorometric test described by Ullrich and Weber (1972). The test system contained in a total volume of 600 μl : 3.3 μM 7-ethoxycoumarin, 3.3 mM MgCl_2 , 0.1 M Tris/HCl buffer pH 7.6 and 16 μl (26.17 pmol CYP2B1 and 52.34 pmol reductase) of the reconstituted enzyme system in a quartz cuvette kept for 3 min at constant 30 °C in a sample holder of the spectrophotometer. The reaction was started by the addition of 16.6 μM NADPH. The rate of umbelliferone production was recorded by the fluorescence change ($\lambda_{\text{E}}=380$ nm; $\lambda_{\text{F}}=460$ nm) versus time. To calibrate each assay 10 μl of a 10^{-4} M solution of umbelliferone was added twice at the end of each experiment.

Energy-transfer-assay of umbelliferone and FMN

To obtain information about the existence or absence of energy transfer between the donor molecule umbelliferone ($\lambda_{\text{E}}=380$ nm; $\lambda_{\text{F}}=460$ nm) and the acceptor molecule FMN ($\lambda_{\text{E}}=460$ nm; $\lambda_{\text{F}}=525$ nm) in the presence of the reconstituted enzyme system (CYP2B1:NADPH-cytochrome P450-reductase) an assay was designed which al-

lows the detection of the fluorescence enhancement of FMN ($\lambda_{\text{F}}=525$ nm) and the fluorescence quenching of the donor molecule umbelliferone ($\lambda_{\text{F}}=460$ nm). The test system contained in a total volume of 600 μl : 3.3 μM 7-ethoxycoumarin, 3.3 mM MgCl_2 , 3.3 μM FMN in 0.1 M Tris/HCl buffer pH 7.6 and 48 μl (78.5 pmol CYP2B1 and 157 pmol reductase) of the reconstituted enzyme system in a quartz cuvette kept for 3 min at constant 30 °C in a sample holder of the fluorometer. The reaction was started by the addition of 16.6 μM NADPH. Exactly at one, five and seven minutes after starting the reaction, the recording of the fluorescence spectrum of the reaction mixture in the range of 390–600 nm ($\lambda_{\text{E}}=380$ nm) was initiated. The same experiment was done without adding FMN or NADPH.

Results

Absorption spectra of the reaction components

The absorption spectra of the substrate 7-ethoxycoumarin, the product umbelliferone, the cosubstrate NADPH and the two flavins FMN and FAD which are the two chromophores of the NADPH-cytochrome P450 reductase are shown in Fig. 1. The absorption coefficients of the 10^{-5} M solutions of the substrate 7-ethoxycoumarin and the cosubstrate NADPH are at $\lambda=380$ nm, zero. But those of the product umbelliferone, FAD and FMN are 6.5, 8.5 and 8.2 $\text{mm}^{-1} \cdot \text{cm}^{-1}$, respectively. Therefore only these chromophores can absorb light at $\lambda=380$ nm. The absorption maxima of FAD and FMN are $\lambda=370$ nm and $\lambda=450$ nm, and the corresponding absorption coefficients are 8.5 and 10 $\text{mm}^{-1} \cdot \text{cm}^{-1}$.

Absorption spectra of the NADPH-cytochrome P450 reductase

The spectra of the NADPH-cytochrome P450 reductase have been deduced previously by Oprian and Coon (1982). Fig. 2 shows four of the nine possible oxydation-reduction states of the reductase (1.0 μM in 0.1 M Tris/HCl buffer, pH 7.6). The absorption maxima and the corresponding absorption coefficients of the protein-bound FAD and FMN in the NADPH-cytochrome P450 reductase are roughly the same compared to the corresponding free oxydized flavins FAD and FMN

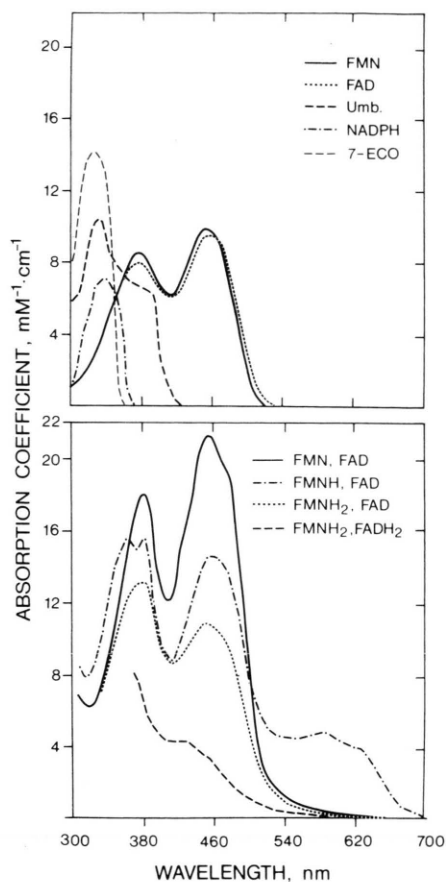


Fig. 1. Absorption spectra and absorption coefficients of the reaction components of the system in 0.1 M Tris/HCl buffer pH 7.6, containing 3.3 mM MgCl_2 . For all components the concentration was 10 μM . 7-ethoxycoumarin = 7-ECO (---); umbelliferone (---); NADPH (---); FMN (—) and FAD (.....).

Fig. 2. Absorption spectra and absorption coefficients for four of the nine possible oxydation-reduction states of the NADPH-cytochrome P450 reductase (1.0 μM in 0.1 M Tris/HCl buffer pH 7.6). The fully oxidized enzyme FMN/FAD (—), the four electrons reduced form of the reductase $\text{FMNH}_2/\text{FADH}_2$ (---), the spectrum of the air-stable semiquinone $\text{FMNH}\cdot/\text{FAD}$ (---) and the two electrons reduced form FMNH_2/FAD (----) were recorded and computed as described by Oprian and Coon (1982).

(see Fig. 1). The flavoprotein with the fully oxydized FMN and FAD per molecule of reductase, has an absorption coefficient $\epsilon_0 = 21 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at $\lambda = 458 \text{ nm}$. At the same wavelength the fully reduced form $\text{FMNH}_2/\text{FADH}_2$ of the reductase has an absorption coefficient $\epsilon_0 = 3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. At 458 nm the absorption-coefficients of the air-stable semiquinone $\text{FMNH}\cdot/\text{FAD}$ and the two-electron reduced form FMNH_2/FAD have absorption coefficients of $\epsilon_0 = 15.5$ and $10.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, respectively.

Difference spectra and Scatchard plot for equilibrium ligand binding

The spectral interaction of umbelliferone with the CYP2B1:NADPH-cytochrome P450 reductase-complex was estimated by difference spectroscopy (Fig. 3). The difference spectra resulting from the addition of increasing amounts of the ligand

umbelliferone (2.5–30 μM) to the preformed CYP2B1 : reductase-complex (0.48 μM :0.58 μM) show absorption peaks at 384 nm and troughs at 414 nm with an isosbestic point at 400 nm. This ligand-induced spectral change is called type I and is typical for compounds binding to the protein-complex near to the catalytic site. A spectral dissociation constant K_s of 20 μM could be gained from the Lineweaver-Burk plot (inset Fig. 3).

Fig. 4 shows the Scatchard plot of the datas, calculated from the difference spectral binding curves shown in Fig. 3. The Scatchard plot of $[\text{U}]_b/[\text{E}]_t \cdot [\text{U}]_f$ (i.e., moles of ligand umbelliferone bound per moles of the total CYP2B1:reductase complex divided by the concentration of free ligand $[\text{U}]_f$) versus $[\text{U}]_b/[\text{E}]_t$ (i.e., moles of ligand bound per mole of enzyme) is shown. The slope of the Scatchard plot ($-1/K_d$) is not linear but curved (Fig. 4). Therefore the enzyme complex possesses multiple independent binding sites with different

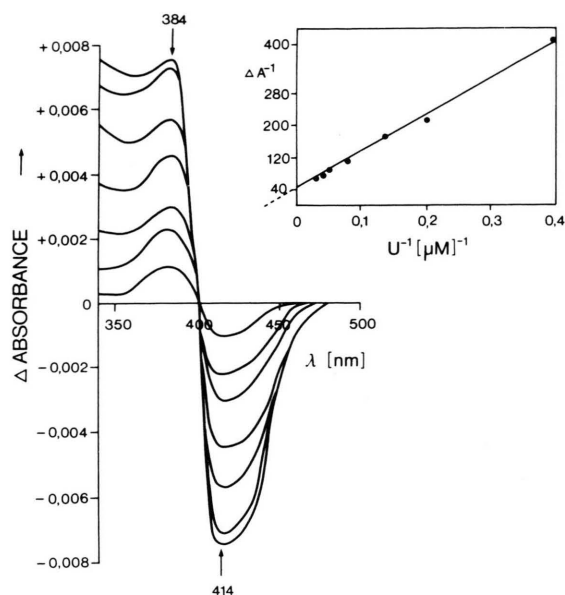


Fig. 3. Difference spectra of increasing amounts of umbelliferone (2.5–30 μM) to the reconstituted preformed complex of CYP2B1:NADPH cytochrome P450 reductase (0.48 μM :0.58 μM). The preformed CYP2B1:reductase-complex was placed in the sample cuvette together with NADPH (0.125 mM) in a total volume of 800 μl 0.1 M Tris/HCl buffer pH 7.6. The reference cuvette contained only NADPH (0.125 mM) in the same 800 μl Tris/HCl buffer. After recording the base line, binding spectra were recorded after each addition of aliquots of a 10^{-3} M Tris/HCl buffer solution of umbelliferone in both cuvettes in the concentration range of 2.5–30 μM . The inset shows the Lineweaver-Burk plot to estimate the spectral dissociation constant $K_s=20 \mu\text{M}$ and the maximal spectral change $\Delta A_{\text{max}}=0.025$ for the ligand umbelliferone (U).

affinities for the ligand umbelliferone. The curve shows a high affinity part and a low affinity part. The intercepts of the tangential lines (----) on the horizontal axis gives n , the number of umbelliferone binding sites per molecule of enzyme. The binding sites of the high affinity part are 12 and those of the low affinity part are more than 34. The intercepts of the same lines (----) on the vertical axis gives n/K_d . K_d (high affinity) = 0.23 μM and K_d (low affinity) = 2.8 μM .

Fluorescence excitation and emission spectra of the donor umbelliferone and the acceptors FMN and FAD

In order to examine whether the fluorescence emission spectrum of umbelliferone overlaps the

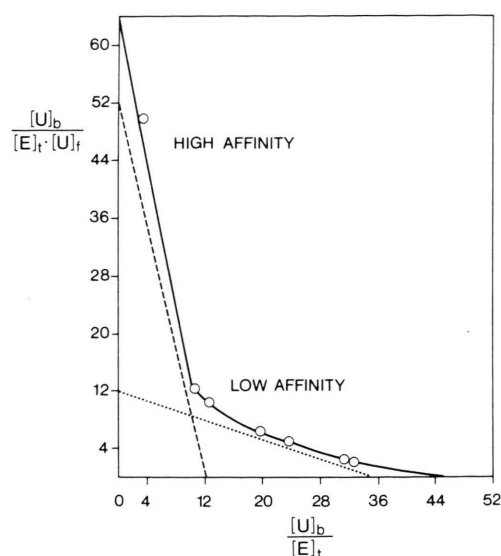


Fig. 4. Scatchard plot to estimate the binding sites n of the ligand umbelliferone (U) per molecule of enzyme (preformed complex of CYP2B1 and NADPH cytochrome P450 reductase). To calculate $[U]_b$, $[U]_f$ and $[E]_t$ data received from the difference spectral binding curves shown in Fig. 3 were used. The Scatchard plot of $[U]_b/[E]_t \cdot [U]_f$ (i.e., moles of ligand umbelliferone bound $[U]_b$ per mole of the total enzyme $[E]_t$, divided by the concentration of free umbelliferone $[U]_f$ versus $[U]_b/[E]_t$ (i.e., moles of umbelliferone bound $[U]_b$ per mole of enzyme) is curved, indicating that the enzyme-complex possesses multiple independent binding sites with different affinities (high affinity and low affinity) for the ligand umbelliferone.

The intercepts of the tangential dotted lines (---) and (----) on the vertical axis gives n/K_d . K_d (high affinity) = 0.23 μM and K_d (low affinity) = 2.8 μM . The intercepts of the same tangential dotted lines on the horizontal axis gives n , the number of umbelliferone binding sites per molecule of the preformed complex. For the high and low affinity part $n = 12$ and $n = 34$, respectively.

fluorescence excitation spectrum of the fluorophore molecules FMN and FAD of the NADPH-cytochrome P450 reductase, fluorescence spectra of these compounds were recorded. Fig. 5 shows the uncorrected fluorescence activation and emission spectra. The donor molecule umbelliferone (0.83 μM) shows $\lambda_{\text{Emax}}=360 \text{ nm}$ and $\lambda_{\text{Fmax}}=458 \text{ nm}$. The acceptor molecules FMN and FAD show both $\lambda_{\text{Emax}}=465 \text{ nm}$ and $\lambda_{\text{Fmax}}=525 \text{ nm}$. The spectral overlap ratio of the fluorescence spectrum of umbelliferone to the excitation spectra of FMN and FAD were calculated to be 1:1.24:0.17, respectively (Fig. 5). Thus the requirement for excitation transfer between appropriate pairs of fluoro-

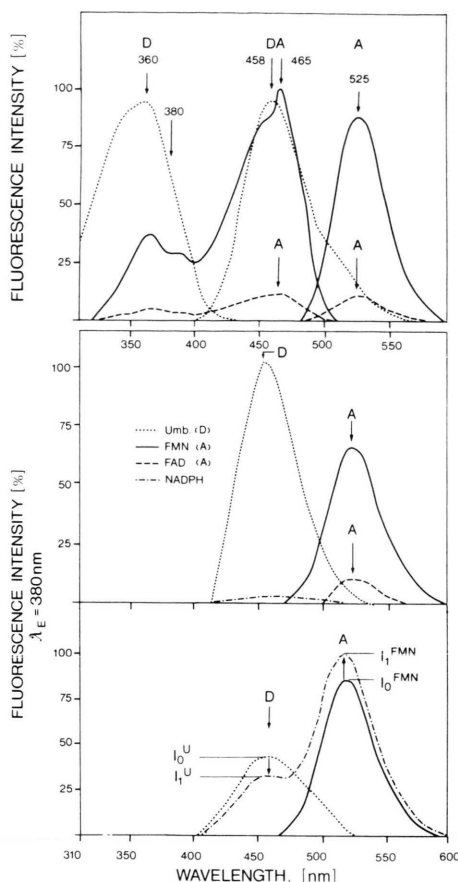


Fig. 5. Uncorrected fluorescence excitation and emission spectra of the donor (D), umbelliferone, and the acceptors (A), FMN and FAD in 0.1 M Tris/HCl buffer pH 7.6, containing 3.3 mM MgCl₂. The wavelength maxima of the excitation and emission spectra were 360 nm and 458 nm for umbelliferone (D) and for the acceptors (A) FMN and FAD 465 nm and 525 nm, respectively. The concentrations used were: 0.83 μ M umbelliferone (-----), 3.3 μ M FMN (—) and 3.3 μ M FAD (---).

Fig. 6. Uncorrected fluorescence emission spectra ($\lambda_E=380$ nm) of the reaction components in 0.1 M Tris/HCl buffer pH 7.6, containing 3.3 mM MgCl₂. The concentrations used were: 0.83 μ M umbelliferone (-----); 3.3 μ M FMN (—); 3.3 μ M FAD (---) and 16.6 μ M NADPH (-·-). The preformed complex, CYP2B1: reductase (0.39 μ M; 0.78 μ M) and the substrate 7-ethoxycoumarin (3.3 μ M) showed no fluorescence under the conditions used.

Fig. 7. Uncorrected fluorescence emission spectra ($\lambda_E=380$ nm) of an incubation of the preformed CYP2B1: reductase-complex (0.39 μ M; 0.78 μ M) with 3.3 μ M 7-ethoxycoumarin, 3.3 μ M FMN, 3.3 mM MgCl₂ and 16.6 μ M NADPH exactly one minute after starting the reaction by the addition of NADPH (-·-). The same reaction without addition of FMN shows curve (-----). And without addition of NADPH shows curve (—). The relative fluorescence quenching at $\lambda_F=465$ nm of the donor (D) umbelliferone (U) is marked by I_0^U and I_1^U . The relative fluorescence enhancement at $\lambda_F=525$ nm of the acceptor (A) FMN is indicated by I_0^{FMN} and I_1^{FMN} . I_1 and I_0 are the fluorescence intensities of FMN or umbelliferone with and without Förster-energy transfer.

phores is given, because the donor fluorescence spectrum overlaps the acceptor excitation spectrum.

Fluorescence spectra of the reaction components under 7-ethoxycoumarin-O-deethylase test conditions

In order to examine the possibility of an electronic excitation energy transfer of the product umbelliferone to the fluorochromes FMN and FAD of the NADPH-cytochrome P450-reductase a 7-ethoxycoumarin-O-deethylase test was designed with very low substrate, cosubstrate (NADPH) and additional FMN and FAD concentrations. Fig. 6 shows the fluorescence spectra of the product umbelliferone (0.83 μ M), the cosubstrate NADPH (16.6 μ M) and FMN (3.3 μ M) and FAD (3.3 μ M). The excitation wavelength was in every case 380 nm. The fluorescence curve of

NADPH do not interfere the fluorescence curves of FMN and FAD at their fluorescence maximum ($\lambda_F=525$ nm). But the procentual interference of the fluorescence intensity of umbelliferone at $\lambda_F=525$ nm is for FMN 6.9% and for FAD 44.4% (Fig. 6). Therefore only the FMN was chosen as an appropriate acceptor for the donor umbelliferone.

CYP2B1-dependent 7-ethoxycoumarin-O-deethylase test

To minimize the interactions of the absorption- and fluorescence-properties of the reaction components shown in Figs 1, 5 and 6, and to prevent an excitation energy transfer from the donor (umbelliferone) to the acceptor (FMN) in the reaction solution, very low concentrations of the substrate 7-ethoxycoumarin (3.3 μ M), and the cosubstrate NADPH (16.6 μ M) were chosen. For the same reason the excitation wavelength of the product

umbelliferone was set to $\lambda_E=380$ nm. Under these conditions described in details under Materials and Methods, the specific 7-ethoxycoumarin-O-deethylase activity was estimated to be $1.0 \text{ nmol umbelliferone} \times \text{min}^{-1} \times \text{nmol CYP2B1}^{-1}$. The umbelliferone increase for three different CYP2B1-concentrations (0.13; 0.26 and $0.39 \mu\text{M}$) in the preformed CYP2B1 : reductase-complex (molar ratio 1:2) was linear for at least seven minutes.

Excitation energy transfer from umbelliferone to FMN under 7-ethoxycoumarin-O-deethylase test conditions

To investigate if in the presence of the CYP2B1: NADPH-P450-reductase protein-complex can happen an electronic excitation energy transfer from the product umbelliferone which can act as donor for the acceptor FMN, 7-ethoxycoumarin-O-deethylase tests were performed with and without the acceptor FMN and with and without the cosubstrate NADPH. Fig. 7 represents the results of the 7-ethoxycoumarin-O-deethylase test as uncorrected fluorescence spectra ($\lambda_E=380$ nm) of an incubation of CYP2B1:reductase-complex ($0.39 : 0.78 \mu\text{M}$) with the substrate 7-ethoxycoumarin ($3.3 \mu\text{M}$) recorded exactly at one minute, after starting the reaction by the addition of cosubstrate NADPH ($16.6 \mu\text{M}$). The fluorescence curve (•••) represents that of the product umbelliferone (U) with $\lambda_{F\text{max}}=465$ nm and the fluorescence intensity I_o^U . Curve (—) represents the fluorescence of FMN with $\lambda_{F\text{max}}=525$ nm and the fluorescence intensity I_o^{FMN} , when the same experiment was performed without the cosubstrate NADPH but by the addition of FMN ($3.3 \mu\text{M}$) to the reaction mixture. When the 7-ethoxycoumarin-O-deethylase test was performed with the addition of FMN ($3.3 \mu\text{M}$) and NADPH ($16.6 \mu\text{M}$) under the same conditions described above, the fluorescence curve (— · —) of the product umbelliferone at $\lambda_{\text{max}}=465$ nm shows a lower fluorescence intensity I_1^U , and the fluorescence curve of FMN at $\lambda_{\text{max}}=525$ nm shows a higher fluorescence intensity I_1^{FMN} , compared to I_o^U and I_o^{FMN} , respectively.

The fact that the $I_1^U < I_o^U$ in the presence of FMN and on the other hand that the fluorescence intensity $I_1^{\text{FMN}} > I_o^{\text{FMN}}$ in the presence of umbelliferone, demonstrates, that umbelliferone acts as a

donor molecule for the acceptor molecule FMN. Obviously a part of the electronic excitation energy ($\lambda_E=380$ nm) of umbelliferone is given radiationless to the acceptor FMN. This observed energy transfer can be explained by the radiationless Förster-transfer (Förster, Th, 1949). One molecule, the donor umbelliferone emits a dipole radiation and when a second molecule, the acceptor FMN is sufficiently close ($R < 50 \text{ \AA}$) to the donor, it can absorb the dipole radiation and no macroscopic radiation (i.e., no fluorescence of the donor molecule) is detectable.

If the radiationless Förster-energy transfer takes place on the surface or in the environment of the catalytic center of the CYP2B1: reductase-complex, it must depend on the concentration of the preformed-complex. Fig. 8 shows the results of the differences of the fluorescence intensities of the product umbelliferone $\Delta I^U/I_o^U$ and those of the acceptor FMN $\Delta I^{\text{FMN}}/I_o^{\text{FMN}}$ as a function of the CYP2B1 concentration. From Fig. 8 one can see a good relationship between the CYP2B1-concentration in the preformed complex and the energy transfer from the donor umbelliferone to the acceptor FMN. These results demonstrate that the

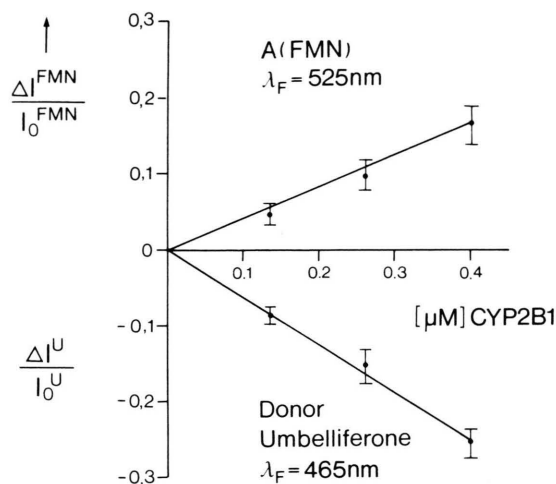


Fig. 8. Relative fluorescence intensity enhancement at $\lambda_F=525$ nm of the acceptor FMN ($\Delta I^{\text{FMN}}/I_o^{\text{FMN}}$) and relative fluorescence intensity quenching at $\lambda_F=465$ nm of the donor umbelliferone ($\Delta I^U/I_o^U$) as a function of the protein concentration of a preformed CYP2B1:NADPH cytochrome P450 reductase-complex. The CYP2B1 concentrations used were: 0.13; 0.26 and $0.39 \mu\text{M}$. The bars indicate the range of three different estimations. The experimental procedure has been described in Fig. 7.

radiationless Förster-transfer is a function of the CYP2B1: reductase-complex concentration.

Discussion

There are two models describing the electron shuttling during the over-all reaction of the cytochrome P450-dependent monooxygenase reaction (Vermilion *et al.*, 1981; Backes, 1993). In the so called two-four-cycle the reductase cycles between the 2-electron-containing reductase (FAD/FMNH_2) and the 4-electron reduced form ($\text{FADH}_2/\text{FMNH}_2$). In the one-three-cycle, however, the reductase cycle occur between the air-stable semiquinone ($\text{FAD}/\text{FMNH}\bullet$) and the 3-electron-containing form ($\text{FADH}\bullet/\text{FMNH}_2$).

In the case of the two-four-cycle the reductase in the working CYP2B1:reductase-complex operates on a higher free energy level ($\Delta G^\circ = +15.5 \text{ kJ/mol}$) than in the one-three-cycle, because the reductase stabilizes the one electron reduced air-stable semiquinone ($\text{FAD}/\text{FMNH}\bullet$) by 180 mV compared with the 2-electron-reduced form FAD/FMNH_2 . This energy can be provided by thermal processes, conformational changes of the protein-complex or by the absorption of light energy (absorbed photons).

The rate-limiting step in the over-all reaction is the transfer of the electron-pair from NADPH to the two-electron reduced reductase FAD/FMNH_2 , to generate the four-electron reduced form $\text{FADH}_2/\text{FMNH}_2$. The rate constant for this reaction was determined by Oprian and Coon (1982) to be 5.4 s^{-1} . The rate constant of electron transfer from the four-electron reduced reductase to the electron acceptor CYP2B4 was found to be $17.5 \cdot \text{s}^{-1}$ (Backes and Reker-Backes, 1988). Therefore, the rate of the oxydation reaction of the four-electron reduced reductase $\text{FADH}_2/\text{FMNH}_2$ by the CYP2B4 is about 3 times ($17.5 \cdot \text{s}^{-1}$) of its production rate from FAD/FMNH_2 by NADPH ($5.4 \cdot \text{s}^{-1}$).

Radda and Melvin Calvin (1964) have determined the rates of photoreduction of FMN and FAD by NADPH by irradiating the reaction mixture with light of wavelength $>400 \text{ nm}$. The rate of photoreduction of FMN was about 6-times faster than that of FAD. This considerable slower rate of photoreduction of FAD was also published by Tether and Turnbull (1962), examining the photo-

reduction of flavins with blue light ($\lambda 340\text{--}450 \text{ nm}$) using ergosterol as hydrogen donor. Their experimental data are consistent with the view, that the triplet state of the excited flavins with its lower energy, longer lifetime (10^{-2}s) and uncoupled spin character (the diradical with its uncoupled electrons at the two nitrogen atoms of the isoalloxazine part) is the chemically reactive intermediate in the photoreduction process. The first excited singlet state (lifetime 10^{-8}s), fluorescent emission ($\lambda_{\text{max}}=525 \text{ nm}$) passes irradiationless to the phosphorescent triplet state ($\lambda_{\text{max}}=605 \text{ nm}$) of flavins (Dhéré and Castelli, 1938). The fluorescence intensity of FAD is about 9 times lower than that of FMN (Fig. 5 and 6) indicating a strong irradiationless transition of the first excited singlet state (FAD') to the triplet state (FAD^*). But this triplet state is also quenched like the singlet state by formation of an internal complex between the isoalloxazine and the adenine parts of the FAD molecule. This internal quenching is not possible in the case of FMN, and therefore the excited diradical triplet state (FMN^*) of the isoalloxazine can complex with the hydrogen donor NADPH to form the dihydroflavin FMNH_2 (Radda and Melvin Calvin, 1964).

The relatively high singlet to triplet transition probability of FAD may contribute to the opening of the internal complex. This, however, terminates the triplet quenching and favours the complexation with an electron donor like NADPH. This was shown by Tether and Turnbull in 1962, who could demonstrate that in the presence of urea, blue light excited FAD is equally fast reduced by an electron donor as FMN. It is obvious that the reactivity of light activated flavins depends mainly on structural and configurational factors.

The two flavins in the native reductase, FMN and FAD, are slightly fluorescent (fluorescence quantum yield $Q_f=0.063$, Bastiaens *et al.*, 1989) indicating that the environment of the isoalloxazine moiety, the neighbouring amino acid residues of the protein, quench significantly the excited singlet state. But the absorption coefficients of the FAD and FMN shown in Figs 1 and 2 are almost the same in free and in the oxidized reductase bound form. The ϵ_0 -values, which reflects the absorbed light energy are highest for the oxidized reductase ($21 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; $\lambda=460 \text{ nm}$) which means that this oxidized form possesses the high-

est probability to be photoreduced with NADPH. The same is valid for the air-stable semiquinone with an ϵ_0 of $15.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at $\lambda=460 \text{ nm}$. The two electron reduced reductase FAD/FMNH₂ has about the same extinction coefficient ($\epsilon_0=10.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) as free FAD (Figs 1 and 2). This signifies that the same light energy can be collected from the protein bounded FAD as from the free FAD. The ability to be activated in the first singlet state and to be stabilized in the triplet state is the same.

As we have shown previously, when blue light is absorbed by the CYP2B1:reductase-complex it leads to the activation of the 7-ethoxycoumarin-O-deethylase (Müller-Enoch and Gruler, 1986; Müller-Enoch, 1994). The low activation effect by direct blue light excitation ($\lambda=440 \text{ nm}$) of the reaction mixture (9% enhancement) compared with the light activation effect on adding the fluorescent product umbelliferone to the reaction and irradiating the reaction mixture with $\lambda_E=365 \text{ nm}$ (93% enhancement), shows, that other effects beside the activation of the two molecules FAD and FMN in the first singlet state must be taken into account. This extremely high autofluorescence activation effect was explained by the assumption that the product umbelliferone is in close contact with the catalytic center of the enzyme-complex and that its irradiation energy provides an additional enhancement of enzyme activity (Müller-Enoch, 1994).

The results in this paper demonstrate, that excitation energy transfer from the donor umbelliferone to the acceptor FMN can occur on the surface of the CYP2B1:reductase enzyme-complex (Figs 7 and 8). This radiationless energy transfer (Förster transfer) can also excite the FAD and FMN molecules in the catalytic center of the protein ($R<50 \text{ Å}$). But this is also the case by an appropriate excitation with blue light ($\lambda_E=440 \text{ nm}$). The decisive difference can be explained by the number of light quanta collected by 12–34 umbelliferone molecules bound per molecule of CYP2B1: reductase-complex (ϵ_0 values at $\lambda=380 \text{ nm}$ of $78\text{--}221 \text{ mM}^{-1} \cdot \text{cm}^{-1}$), centered in the FAD-binding domain. This, however, results in an excited state of the protein-FAD moiety in such a way that by conformational changes,

the amino acid residues responsible for the quenching of the fluorescent first singlet state and triplet state are fluctuated so, that the chemically active triplet state of the isoalloxazine part is not quenched by its adenine part and can complex with the NADPH, to form the dihydroflavine FADH₂.

The assumption of such mechanism is reasonable when the activation energy of the light reaction is lower than that of the dark reaction. The energy difference of the first excited singlet state and the triplet state can be calculated by the wave-number differences $\Delta\nu$ of its fluorescence ($\lambda_{\text{max}}=525 \text{ nm} = 19048 \text{ cm}^{-1}$) and the β -phosphorescence ($\lambda_{\text{max}}=605 \text{ nm} = 16529 \text{ cm}^{-1}$) to give for the triplet state a $7.2 \text{ kcal/mol} = 30 \text{ kJ/mol}$ lower activation energy. A comparison of the activation energies, obtained from Arrhenius plots for the CYP2B1-dependent 7-ethoxycoumarin-O-deethylase with and without umbelliferone irradiation (light reaction, dark reaction) provides 14.7 kJ/mol and 41.9 kJ/mol , respectively. The difference of 27.2 kJ/mol (Müller-Enoch, 1994) is very close to the calculated value of 30 kJ/mol for the energy difference of the singlet and triplet states of FAD.

Therefore, it is most likely to accept that the fluorescence energy of 12–34 umbelliferone molecules (U) transfer their energy to the FAD in the catalytic center radiationless. This leads to an activation of the first singlet state of FAD' and to a conformational change of the FAD-binding domain favouring an encaging of the FAD* triplet state and complexation with NADPH generates the FADH₂. The result is that the reaction of the FAD* in the triplet state is by 30 kJ/mol lower in energy than the dark reaction. The entire process described above can be summarized in the following scheme

1. $\text{U} + \text{FAD} + h \cdot \nu_E \rightarrow \text{U}' + \text{FAD} \rightarrow \text{U} + \text{FAD}'$
2. $\text{FAD}' \rightarrow \text{FAD}^*$ (singlet-triplet-transition)
3. $\text{FAD}^* + \text{NADPH} + \text{H}^+ \rightarrow \text{FADH}_2 + \text{NADP}^+$

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