Product Autofluorescence Activation in the Cytochrome P 450 Monooxygenase System

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Cytochrome P450, Blue Light Activation, Photoreceptor-Phototransducer, Umbelliferone, Scopoletin

The relative increase of the 7-ethoxycoumarin-O-deethylase and the 6,7-dimethoxycoumarin-O-demethylase activities were found 93 and 236% using a reconstituted cytochrome P4502B1:NADPH-P450 reductase system by adding to the reaction mixtures their own products. The assays were irradiated during the reactions with the excitation wavelength maximum of their products umbelliferone (λ_E = 365 nm) or scopoletin (λ_E = 398 nm), respectively. Addition of the products to the reaction mixtures without irradiation (dark reaction) had no activating effect on the specific activities of the 7-ethoxycoumarin-O-deethylase or the 6,7-dimethoxycoumarin-O-demethylase. The relative increase of the specific activities is dependent on the excitation light intensities and was at maximum when the light intensity at the sample cuvette was 0.4 mW/cm^2.

The activation energies of the P4502B1-dependent 7-ethoxycoumarin-O-deethylation reaction obtained from Arrhenius plots with and without added umbelliferone and irradiation with λ_E = 365 nm are 14.7 kJ/mol and 33.5 kJ/mol, respectively, in the temperature range of 27–37 °C. The irradiation energy of the fluorescent product umbelliferone change the catalytic mechanism, which has a two times lower activation energy in the presence of the irradiated product umbelliferone.

Umbelliferone and scopoletin have highest fluorescence intensities in the wavelength range of the blue light (440–480 nm). The photochemical action spectrum of the 7-ethoxycoumarin-O-deethylase of the P4502B1:reductase system is also found to be in the wavelength range of 420–470 nm. No activation effect was seen with irradiating light lower than 400 nm. Obviously the fluorescence light of the products are due for the extremely high activation effect. This is the first report that the products umbelliferone and scopoletin are photoreceptors and phototransducers.

Introduction

The activation of the cytochrome P450-dependent monooxygenase system by light was first published by Müller-Enoch and Gruler (1986). The relative increase of the 7-ethoxycoumarin-O-deethylase activity of the reconstituted P4502B1:NADPH-P450 reductase system by irradiating the system with a second light source with light of 440 nm was 9%. The photochemical action spectrum for the reaction was in the wavelength range of 400–470 nm.

In this paper it will be shown, that the light-induced enhancement of the monooxygenase activity is extremely increased (93%) when the irradiation light comes directly from the product umbelliferone. Umbelliferone (P) is activated by a wavelength of 365 nm (ν_E) and it fluoresces at a wavelength of 453 nm (ν_F).

$P + h \cdot \nu_E \rightarrow P^* \rightarrow P + h \cdot \nu_F$. This product fluorescence light irradiates the active species in the catalytic center directly, because it is in close contact with the active center of the enzyme.

Abbreviations: P4502B1, liver microsomal cytochrome P450; recommended nomenclature of Nebert *et al.* (1991). PB, phenobarbital.

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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. 6,7-Dimethoxycoumarin (scoparone) and 7-hydroxy-6-

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methoxycoumarin (scopoletin) were purchased from Carl Roth GmbH (Karlsruhe, Germany), and recrystallized from water. 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). All materials used for the purification procedure of the cytochrome P4502B1 and the NADPH-cytochrome P450 reductase are described by Guengerich *et al.* (1982).

Animals and induction procedure

In all experiments male Sprague-Dawley rats, weighing between 200–250 g, which were allowed free access to food and water were used. Treatment with phenobarbital (PB) consistent of the addition of 0.1% (w/v) PB to the drinking water for 6 days prior to sacrifice. Livers were removed and placed in ice-cold 0.25 M sucrose.

Purification of cytochrome P4502B1 and of the NADPH-cytochrome P450 reductase

Rat liver cytochrome P4502B1 (apparent monomeric M_r = 50,000) and NADPH-cytochrome P450 reductase (M_r = 74,000) were purified to electrophoretic homogeneity out of microsomal fractions prepared from PB-treated rats using procedures described by Guengerich and Martin (1980). The SDS-PAGE pure P4502B1 and the reductase had specific contents of 17.02 nmol/ml and 28.2 nmol/ml, respectively. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Spectrophotometry

The cytochrome P450 content was determined by recording the CO-reduced minus reduced difference spectra according to Omura and Sato (1964) using an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$. The NADPH-cytochrome P450 reductase activity was determined according to Yasukochi and Masters (1976) and expressed as NADPH-cytochrome *c* reductase activity at 30 °C in 0.3 M potassium phosphate buffer, pH 7.7. Absolute spectra and difference spectra of the pure P4502B1 and the reductase were recorded from 370–600 nm using a Varian Cary spectrophotometer model 219 (Varian GmbH, Darmstadt, Germany).

Reconstitution of the P4502B1 with the NADPH-cytochrome P450 reductase

The purified cytochrome P4502B1 was mixed with the NADPH-P450 reductase in a 1:2 molar ratio and diluted with 0.1 M Tris-chloride buffer, pH = 7.6 to yield final concentrations of P4502B1 and reductase of 1.89 and 3.78 μM, respectively. This reaction mixture were allowed to stand for 1–1.5 h at room temperature as described by Müller-Enoch (1993). Aliquots of this enzyme mixture (16 μl = 30.26 pmol P4502B1 and 60.52 pmol reductase) were used for all experiments.

Fluorometry

The fluorescence activation and emission spectra of 7-ethoxycoumarin, umbelliferone (7-hydroxycoumarin), NADPH and the reconstituted enzymes (P450 and reductase) were recorded with a Jobin Yvon spectrofluorometer, model JY3D Instruments S.A. (Unterhaching, Germany) in quartz cuvettes of 10 mm light path; Type 104F-QS from Hellma (Freiburg, Germany). The optical band width was 4 nm. The uncorrected excitation and emission spectra of all substances in 0.1 M Tris-(hydroxymethyl)-aminomethane-chloride buffer (Tris-chloride buffer), pH 7.6 were recorded in the wavelength range from 300–540 nm, respectively.

Fluorometric determination of the reconstituted P450:reductase enzyme activity with 7-ethoxycoumarin as substrate

The 7-ethoxycoumarin-O-deethylase activity of the reconstituted P4502B1: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: 0.1 mM 7-ethoxycoumarin, 3.3 mM $MgCl_2$, 0.1 M Tris-chloride buffer, pH = 7.6 and 16 μl (30.36 pmol P450 and 60.52 pmol reductase) of the reconstituted enzyme system in a quartz cuvette kept for 3 min at constant 27 °C in a sample holder of the spectrofluorometer. The reaction was started by the addition of 0.166 mM NADPH. The rate of the umbelliferone production was recorded by the fluorescence change (λ_E = 365 nm; λ_F = 460 nm) *versus* time. To calibrate each assay 10 μl of a 10^{-4} M solution of um-

belliferone was added twice at the end of each experiment.

Fluorometric determination of the reconstituted P450:reductase enzyme activity with 6,7-dimethoxycoumarin (scoparone) as substrate

The 6,7-dimethoxycoumarin-O-deethylase activity of the reconstituted P4502B1:NADPH-P450 reductase system was assayed using the direct fluorometric test described by Müller-Enoch *et al.* (1981). The system contained in a total volume of 600 µl: 50 µM 6,7-dimethoxycoumarin, 3.3 mM $MgCl_2$, 0.1 M Tris-chloride buffer, pH = 7.6 and 16 µl (30.36 pmol P450 and 60.52 pmol reductase) of the reconstituted enzyme system in a quartz cuvette kept for 3 min at 27 °C. After the reaction was started by the addition of 0.166 mM NADPH, the rate of the 7-hydroxy-6-methoxycoumarin (scopoletin) production was recorded by the fluorescence change (λ_E = 398 nm; λ_F = 460 nm) *versus* time. To calibrate each assay 10 µl of a 10^{-4} M solution of scopoletin was added twice at the end of each experiment.

Irradiation of the 7-ethoxycoumarin-O-deethylase test system with the excitation wavelength of the product umbelliferone (λ_E = 365 nm)

The irradiation of the reaction mixtures was performed in the same fluorescence spectrophotometer described above. It has a Xenon lamp as activation light source XBO 150 W/1 from Osram (München, Germany). The excitation wavelength was set to 365 nm and the fluorescence wavelength to 460 nm, respectively, The light intensity of the irradiation at 365 nm could be varied by use of different grey filters in the excitation wavelength pathway and by the use of a shutter for the dark reaction. The light intensity at the position of the sample was measured as described by Häberle *et al.* (1990) and was 0.4 mW/cm^2 using an optical slit of 4 nm spectral band width and the excitation wavelength of 365 nm. The reaction mixtures contained in a total volume of 600 µl 0.1 M Tris-chloride buffer: 13.33 µM 7-ethoxycoumarin; 3.33 µM or 6.67 µM umbelliferone; 3.3 mM $MgCl_2$ and 16 µl (30.36 pmol P450 and 60.52 pmol reductase) of the reconstituted enzyme system thermostated for 3 min at 27 °C in a quartz cuvette. The reaction was started by the addition of 0.167 mM NADPH. The formation of umbelliferone was monitored fluorometrically (λ_E = 365 nm; λ_F = 460 nm) as a function of time.

Irradiation of the 6,7-dimethoxycoumarin-O-demethylase test system with the excitation wavelength of the product scopoletin (λ_E = 398 nm)

The irradiation of the reaction mixture was performed in the same way described for the 7-ethoxycoumarin test. The excitation wavelength of scopoletin (Müller-Enoch *et al.*, 1981) was set to 398 nm and the fluorescence wavelength to 460 nm, respectively. The reaction mixtures contained in a total volume of 600 µl 0.1 M Tris-chloride buffer: 50 µM 6,7-dimethoxycoumarin; 6.67 µM scopoletin; 3.3 mM $MgCl_2$, and 16 µl (30.36 pmol

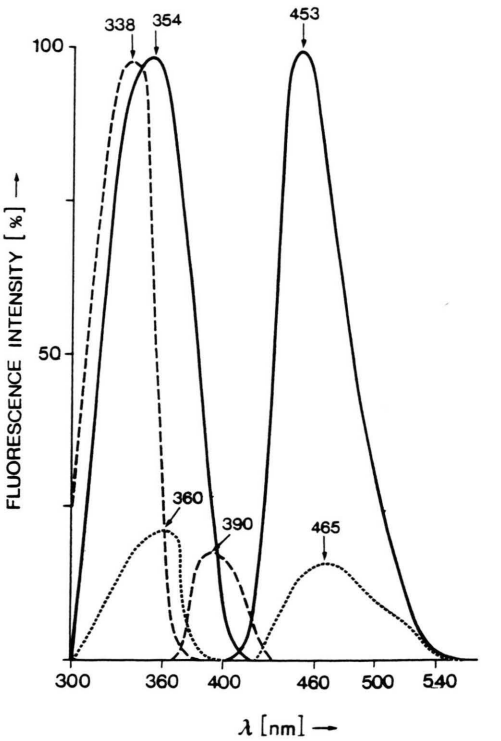

Fig. 1. Uncorrected fluorescence activation and emission spectra of the reaction components of the system in 0.1 M Tris/HCl buffer, pH 7.6, containing 3.3 mM $MgCl_2$. For all components the monitored wavelength of excitation and emission spectra were 365 nm and 460 nm, respectively. The spectra were recorded in the ratio mode. The concentrations used were: 50 µM 7-ethoxycoumarin (– – –), 0.167 mM NADPH (····) and 3.33 µM umbelliferone (——). The reconstituted enzyme system shows no fluorescence under the same conditions.

P450 and 60.52 pmol reductase) of the reconstituted enzyme system thermostated for 3 min at 27 °C in a quartz cuvette. The product scopoletin was monitored fluorometrically (λ_E = 398 nm; λ_F = 460 nm) as a function of time.

Results

Fluorescence spectra of the reaction components under test conditions

Fig. 1 shows the results of the uncorrected fluorescence activation and emission spectra of the substrate 7-ethoxycoumarin (50 μM), the co-substrate NADPH (0.167 mM) and the product umbelliferone (7-hydroxycoumarin) (3.33 μM) monitored in a 0.1 M Tris-chloride buffer, pH = 7.6, containing 3.3 mM $MgCl_2$. The excitation wavelength was λ_E = 365 nm and the fluorescence wavelength was λ_F = 460 nm. These are the wavelengths used for the direct fluorometric P450 activity assay with 7-ethoxycoumarin as a substrate. The substrate 7-ethoxycoumarin shows at a high concentration (50 μM) no fluorescence intensity at 460 nm. For the co-substrate NADPH (0.167 mM) the fluorescence intensity at 460 nm is 20% of the 100% fluorescence intensity of the umbelliferone at the very low concentration 3.3 μM. In a steady-state reaction with 7-ethoxycoumarin as a substrate, the product umbelliferone will increase and the co-substrate NADPH will decrease with time. Therefore, the fluorescence intensity increase at λ_F = 460 nm is proportional to the umbelliferone concentration enhancement when setting the fluorescence intensity at the start of the reaction to zero.

For umbelliferone the fluorescence maximum is λ_F = 453 nm and in the wavelength range of λ = 440–460 nm it shows 90% of its maximal fluorescence intensity.

Steady-state spectral investigation of the reconstituted P4502B1:NADPH-P450 reductase system during catalytic turnover

The microsomal cytochrome P4502B1 metabolize *d*-benzphetamine to a product which bind ferrous P450 and form a spectral complex at λ_{max} = 455 nm as shown by Werringloer and Estabrook (1973) during the aerobic steady state of benzphetamin metabolism. Fig. 2 shows the results when the same experiment was done with the reconstituted cytochrome P4502B1:NADPH-P450 reductase with 7-ethoxycoumarin as a substrate. The spectra shown in Fig. 2 represents the repetitive scanning of difference spectra of the oxidative metabolism of the substrate 7-ethoxycoumarin at an initial concentration of 50 μM in both, the sample and the reference cuvettes. The first tracing (labelled 1 min) after adding of NADPH to the sample cuvette in a concentration of 400 μM shows no absorption band with a maximum at about 450 nm. Repetitive scanning at intervals of 2 min revealed no absorption band with a maximum around 450 nm. But after 17 min when the product concentration of umbelliferone is about

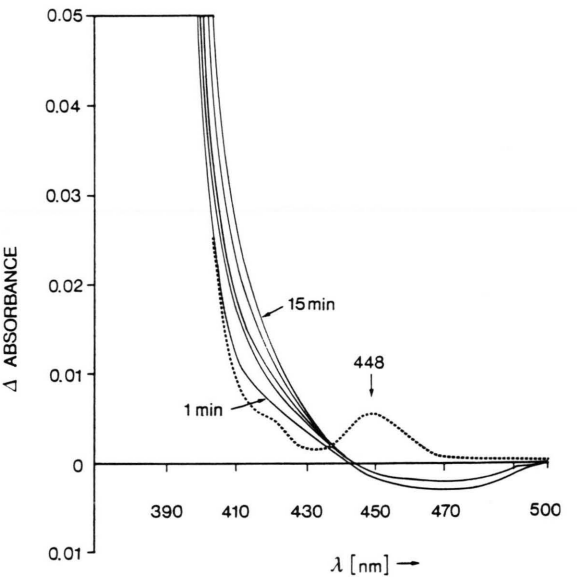

Fig. 2. Repetitive scanning of difference spectra of P4502B1 and reductase during the oxidative metabolism of 7-ethoxycoumarin. A solution of P4502B1 (0.272 μM), NADPH-P450 reductase (0.564 μM), 7-ethoxycoumarin (50 μM), $MgCl_2$ (3 mM) in a total volume of 960 μl 0.1 M Tris-chloride buffer (pH 7.6) were placed in a sample and a reference cuvette. After recording the base line at 27 °C the reaction was started by the addition of 40 μl of NADPH (400 μM) to the sample cuvette. The changes with time of the difference spectra were recorded by repetitive scanning after 1, 3, 5, 7, 9, 11 and 15 min. The first and the last scan recorded as solid lines are labelled 1 and 15 min. 17 min after initiating the reaction by the addition of NADPH to the sample cuvette, the P450 and the reductase were reduced fully in both, the sample, and the reference cuvette by addition of a few grains of solid sodium dithionite, and the resulting difference spectrum was recorded (dashed line).

40 μM in the sample cuvette and the reduction of the P450:reductase complex is completed by the addition of $Na_2S_2O_4$ to both cuvettes, the recorded difference spectrum shows an absorption maximum at 448 nm which is best interpreted as a ferrous cytochrome P450 complex with the product umbelliferone, because it is a lipophilic compound with unoccupied electron pairs which produce complexes with ferrous cytochrome having absorption maxima around 450 nm (Ullrich and Schnabel, 1973; Werringloer and Estabrook, 1973; Estabrook *et al.*, 1971).

7-Ethoxycoumarin-O-deethylase activity of P4502B1 as a function of substrate and product concentrations

In Fig. 3 the results of the 7-ethoxycoumarin-O-deethylation activities of the reconstituted P4502B1:reductase system in the concentration range of 6.66–200 μM of 7-ethoxycoumarin is given in a Lineweaver-Burk plot. The apparent K_m and V_{max} are 55 μM and 25 nmol umbelliferone/min×nmol P450, respectively. When 3.33 or 6.66 μM umbelliferone are added to the reaction mixtures and the umbelliferone production is monitored as usual by the direct fluorometric assay described (λ_E = 365 nm; λ_F = 460 nm) the specific 7-ethoxycoumarin-O-deethylase activities are higher than those of the controls and are also concentration-dependent of the added product umbelliferone (6.66 μM higher than 3.33 μM). No great change in the apparent V_{max} values is observed.

The influence of the product umbelliferone on the fluorescence intensity of the P4502B1-dependent 7-ethoxycoumarin-O-deethylase activity

In order to examine whether the enhancement of the 7-ethoxycoumarin-O-deethylase activity by the addition of umbelliferone is due to a product of umbelliferone with the same excitation and fluorescence maxima of umbelliferone, the reconstituted P450:reductase system was incubated with umbelliferone alone. Fig. 4 shows the result of this experiment. There is no fluorescence intensity increase with the reaction time. Therefore the product umbelliferone did not enhance the P4502B1-dependent 7-ethoxycoumarin-O-deethylase activity by a second product which has

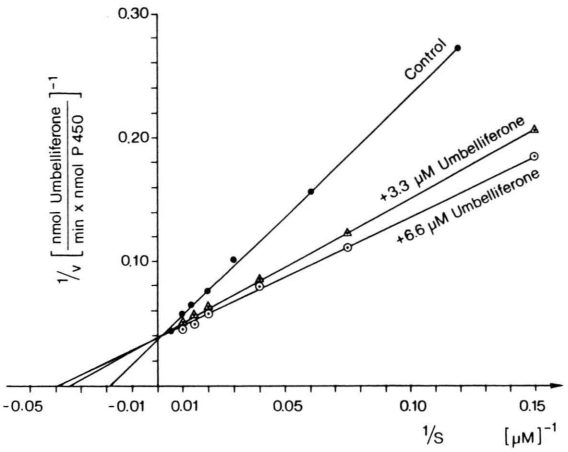

Fig. 3. Lineweaver-Burk plots of reconstituted cytochrome P4502B1:NADPH-cytochrome P450 reductase systems with varying concentrations of 7-ethoxycoumarin (6.66–200 μM) in the absence of umbelliferone (●—●) and in the presence of 3.3 μM (△—△) and 6.6 μM (○—○) umbelliferone. Apparent K_m (control) = 55 μM; K_m (3.3 μM umbelliferone) = 29 μM; K_m (6.6 μM umbelliferone) = 25 μM.

the same fluorescence maximum as umbelliferone itself.

Effects of irradiated light intensities, wavelengths, and added products on the enhancement of the specific enzyme activities

The effect of the irradiated light intensity on the specific 7-ethoxycoumarin-O-deethylase activity in the presence of added umbelliferone is shown in Table I. When setting the spec. activity of the dark reaction (reaction in the cuvette by the use of a shutter in the excitation pathway) to 100%, all other spec. activity measurements with different excitation light intensities in the range of 0.4–0.02 mW/cm² are higher than 100%, namely 193–113%, respectively. These experiments clearly indicates the activation of the enzyme system by light with the excitation wavelength maximum (365 nm) of its product umbelliferone and its dependence on the irradiated light intensity.

In order to show that the fluorescence light at 460 nm (second light effect) and not the excitation light at 365 nm (first light effect) of umbelliferone accounts for the enhancement of the enzyme activity, the same experiments were carried out with

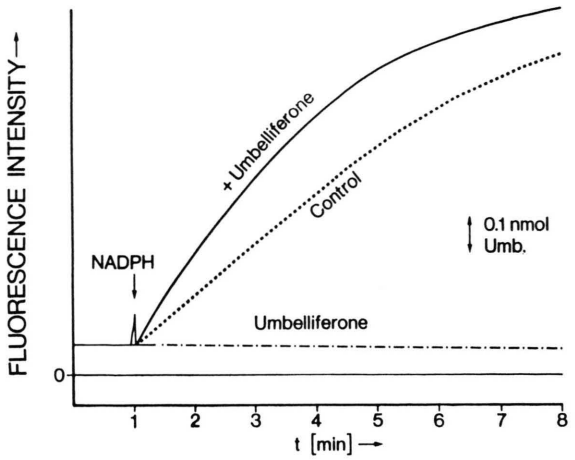

Fig. 4. The effect of the product umbelliferone on its own formation by a reconstituted cytochrome P4502B1: NADPH-cytochrome P450 reductase system with 7-ethoxycoumarin as substrate. The reaction mixtures containing in a total volume of 600 µl of 0.1 M Tris/HCl buffer, pH 7.6: 13.33 µM 7-ethoxycoumarin; 3.33 mM $MgCl_2$ and 16 µl (30.36 pmol P450 and 60.52 pmol reductase) of the reconstituted P4502B1:reductase system thermostated at 27 °C. The reactions were started by addition of NADPH (0.167 mM) as indicated by the arrow. The formation of umbelliferone was monitored fluorometrically (λ_E = 365 nm; λ_F = 460 nm) as a function of time. The curve-labelled control (– – –) contains the complete reaction mixture, curve + umbelliferone (——) contains additional 6.66 µM umbelliferone and the straight line-labelled umbelliferone (—●—) showing no fluorescence change with time, was obtained without substrate but with 6.66 µM of the product umbelliferone.

another substrate which is metabolized by the reconstituted monooxygenase system to a product, which has the same fluorescence but another excitation wavelength than umbelliferone.

Table I summarizes the results of the light intensity-dependent 6,7-dimethoxycoumarin-O-demethylase activities in the presence of added product scopoletin. The specific activities by irradiation the reaction mixture with the excitation wavelength of scopoletin at 398 nm show a strong light intensity dependence in the range of 0.4–0.02 mW/cm^2. The activation effect is in this range 336–133% of a 100% specific activity of the dark reaction.

These results demonstrate clearly, that different excitation wavelengths of the products umbelliferone and scopoletin (365 nm and 398 nm) generates the same activating light effect because both products have the same fluorescence maximum at 460 nm.

Dependence of the 7-ethoxycoumarin-O-deethylase activity upon temperature in the absence and presence of irradiated umbelliferone

The dependence of the P4502B1-dependent 7-ethoxycoumarin-O-deethylase activity with temperature in the absence and presence of umbelliferone (λ_E = 365 nm) is shown in Fig. 5. The Arrhenius plots of the ln of the specific enzyme activity *versus* the inverse of the absolute temperature were different. For all temperatures examined the spec. activities were higher by addition of umbelliferone and irradiation. With umbelliferone the plot show a definite change in slope at 27 °C indicating, that at this temperature the reaction rate changes from one rate-limiting step to another. Without umbelliferone there is a slight

Table I. Effects of irradiated light intensities and added products umbelliferone or scopoletin on the 7-ethoxycoumarin O-deethylase or the 6,7-dimethoxycoumarin O-demethylase activity of a reconstituted cytochrome P4502B1:NADPH-P450 reductase system. Excitation and fluorescence wavelengths of umbelliferone: λ_E = 365 nm; λ_F = 460 nm and scopoletin: λ_E = 398 nm; λ_F = 460 nm. The light intensity of the excitation pathway at the sample cuvette was 0.40 mW/cm^2 for the optical slit of 4 nm spectral band width. The substrate concentrations for 7-ethoxycoumarin and 6,7-dimethoxycoumarin were 13.33 µM or 50 µM, respectively, and the product concentrations for umbelliferone and scopoletin were both 6.67 µM.

Spec. activity $\left[\frac{\text{nmol umbelliferone}}{\text{min} \times \text{nmol P450}}\right]$	Activity [%]	Spec. activity $\left[\frac{\text{nmol scopoletin}}{\text{min} \times \text{nmol P450}}\right]$	Activity [%]	Excitation light intensity at the sample cuvette [mW/cm^2]
5.47	100	1.43	100	0.00 (Shutter)
10.56	193	4.81	336	0.40
8.45	154	3.20	224	0.23
7.81	143	2.80	196	0.14
6.20	113	1.90	133	0.02

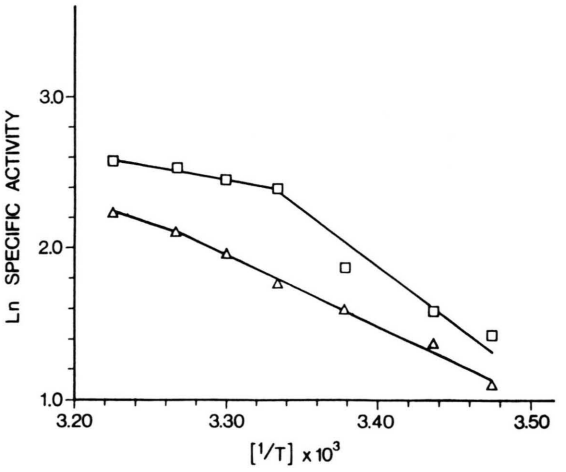

Fig. 5. Dependence of P4502B1-dependent 7-ethoxycoumarin O-deethylase activity upon temperature in the presence and absence of irradiated umbelliferone. The assay media contained in a total volume of 600 µl 0.1 M Tris-chloride buffer (pH 7.6): 13.33 µM 7-ethoxycoumarin, 3.33 mM $MgCl_2$ and 16 µl (30.36 pmol P450 and 60.52 pmol reductase) of the reconstituted P4502B1 reductase system in a quartz cuvette thermostated for 3 min at the different temperatures used and irradiated with λ_E = 365 nm. All reactions were started by the addition of 0.166 mM NADPH, either in the absence (△—△) or in the presence of 6.66 µM umbelliferone. With umbelliferone (□—□) the plot show a definite change in slope at 27 °C. The apparent activation energies were calculated to be 14.7 kJ/mol and 58.6 kJ/mol in the regions of 27 to 37 °C and 17 to 27 °C, respectively. Without umbelliferone (△—△) but for the same temperature regions the apparent activation energies were calculated to be 33.5 kJ/mol and 41.9 kJ/mol, respectively.

slope change at 33 °C. For reactions with umbelliferone (□—□) the apparent activation energies calculated from the Arrhenius equation for the two temperature regions of 27–37 °C and 17–27 °C, are 14.7 kJ/mol and 58.6 kJ/mol, respectively. And for reactions without umbelliferone (△—△), the activation energies were calculated to be 33.5 kJ/mol (27–37 °C) and 41.9 kJ/mol (27–17 °C), respectively. These results show, that the activation energy for the 7-ethoxycoumarin-O-deethylase reaction is drastically reduced in the temperature region of 27–37 °C by the addition of its own fluorescent product when irradiated with its excitation wavelength of 365 nm.

Discussion

The sodium dithionite-reduced difference spectrum of the steady-state experiment shown in Fig. 2 revealed the appearance of a spectral species with an absorption band maximum at 448 nm. This spectral species is best explained by the general excepted assumption, that lipophilic compounds with unoccupied electron pairs will produce complexes with the ferrous cytochrome P450 having Soret absorptions around 450 nm (Ullrich and Schnabel, 1973; Werringloer and Estabrook, 1973; Estabrook *et al.*, 1971). The product umbelliferone is a lipophilic compound and bind to the active site of the enzyme in status nascendi. Therefore the light-induced enhancement of the enzyme activity may be explained with the photodissociation of a ferrous iron-product complex with light of 448 nm.

In order to evaluate K_i values for the P4502B1-dependent 7-ethoxycoumarin-O-deethylase activity with different substrate and product concentrations by use of the direct fluorometric assay, we gained the surprising results shown in Fig. 3. The product umbelliferone activates the reaction, and the degree of the activation is higher by lowering the substrate concentration and enhancing the product concentration. What happens in the cuvette, when the reconstituted system catalyzed the O-deethylation of the substrate 7-ethoxycoumarin? The substrate is deethylated to the product umbelliferone (7-hydroxycoumarin) which fluoresces time-dependent (Fig. 4) by its own fluorescence at λ_F = 453 nm, when activated by its excitation wavelength of λ_E = 365 nm. Or in other words, in the cuvette is an increase in blue light. The fluorescence curve of the product umbelliferone has a maximum at λ_F = 453 nm and its relative fluorescence intensity in the range of 440–460 nm is 90% of its maximum (Fig. 1). Therefore the fluorescence light of the product umbelliferone in the reaction cuvette serves not only as "detection light", but also as "irradiation light" in the wavelength region of 440–480 nm (blue light) and activates all molecules of the intermediate states of the catalytic cycle which have absorption maxima in the region of 450 nm, and which are rate limiting.

If the blue fluorescence light of the product umbelliferone is due for the enhancement of the

specific enzyme activity it must be light intensity-dependent. The results in Table I shows this dependency for four different light intensities and for the dark reaction. Without fluorescence light of the product umbelliferone the specific 7-ethoxycoumarin-O-deethylase activity was set to 100%. With the light intensity of 0.4 mW/cm^2 at the sample cuvette a value of 195% is received.

The same activation effect (Table I) is seen for another substrate 6,7-dimethoxycoumarin, which is metabolized by the reconstituted enzyme system to the fluorochrome product scopoletin (Müller-Enoch *et al.,* 1981, 1984). Scopoletin has nearly the same fluorescence spectrum like umbelliferone, but a different excitation spectrum with a maximum at 398 nm (Müller-Enoch *et al.,* 1981). The results in Table I demonstrates, that light of two different excitation wavelengths (365 nm, 398 nm) generates the same extremely high activation effect on the reconstituted monooxygenase system. This light-induced activation is best explained by the assumption, that the two products umbelliferone and scopoletin are in close contact with the catalytic center and their fluorescence light irradiates the active species in the hydrophobic environment of the polypeptide chain directly and may change the catalytic mechanism by affecting the conformational dynamics in substrate binding and product release.

The high activating effect of the blue fluorescence light on the enzyme-catalyzed reaction was studied by different reaction temperatures in the absence and presence of added umbelliferone to evaluate the activation energies of the reactions. As reported earlier by Müller-Enoch *et al.* (1984) the energy of activation for the P4502B1-dependent 7-ethoxycoumarin-O-deethylase activity was approximately 58.6 kJ/mol without added umbelliferone. A comparison of the activation energies for the same reaction, obtained from Arrhenius plots shown in Fig. 5, revealed, that in the temperature range of 27–37 °C the activation energy with umbelliferone is more than two times lower (14.7 kJ/mol) than without umbelliferone (33.5 kJ/mol). These results demonstrate, that the irradiation energy of the product umbelliferone changes the catalytic mechanism which has a lower activation energy.

The activation of the cytochrome P4502B1-dependent 7-ethoxycoumarin-O-deethylase activity with light at 440 nm was reported to be about 9% (Müller-Enoch and Gruler, 1986). In this experiments an external light source irradiates the reaction mixture. The activation light has to reach the catalytic heme-containing center. The heme is deeply burried and exists in a hydrophobic environment of the polypeptide chain. The light absorption changes the state of the molecule (P450 ground state $\xrightarrow{h \cdot \nu}$ P450 excited state) and in the excited state the specific enzyme activity should be higher than in the non-excited state.

In this paper it is shown how the light source can be brought in close contact with the catalytic center of the enzyme by irradiating the product molecules with its excitation light. One could say the product molecules umbelliferone or scopoletin are the light antenna (first light effect) and light transformer ($\lambda_E \rightarrow \lambda_F$) and concentrator (second light effect) adjusted to the absorption of the active species in the monooxygenase-catalyzed reaction directly, and thus the specific enzyme activity is dramatically increased.

From the results of the experiments carried out in this paper it is concluded, that the autofluorescence light (blue light)-catalyzed monooxygenase reaction with the cytochrome P450 and the flavoprotein NADPH-P450 reductase play an essential role for species which are exposed to light (sun light, artificial light). For humans and animals targets are the skin, eyes or surface.

In plants, where the P450-dependent monooxygenase system is demonstrated to catalyze the O-dealkylation of the substrate 7-ethoxycoumarin to umbelliferone (Werck-Reichhart *et al.,* 1990), this reaction represents a blue light activation of the enzyme system during light irradiation. For plants it is predicted, that also other enzyme-catalyzing reactions which produce fluorochromes (fluorescent products) from fluorogens (non-fluorescent substrates) for example phosphatases, esterases, glycosidases, sulfatases and hydroxylases may be also activated by the fluorescence light energy of their own products.

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Estabrook R. W., Hildebrandt A. G., Baron J., Netter K. J. and Leibman K. (1971), A new spectral intermediate associated with cytochrome P450 function in liver microsomes. Biochem. Biophys. Res. Commun. **42,** 132–139.

Guengerich F. P. and Martin M. V. (1980), Purification of cytochrome P450, NADPH-cytochrome P450 reductase, and epoxide hydratase from a single preparation of rat liver microsomes. Arch. Biochem. Biophys. **205,** 365–379.

Guengerich F. P., Dannan G. A., Wright S. T., Martin M. V. and Kaminsky L. S. (1982), Purification and characterization of liver microsomal cytochromes P450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. Biochemistry **21,** 6019–6030.

Häberle W., Gruler H., Dutkowski Ph. and Müller-Enoch D. (1990), Light-induced activation and synchronization of the cytochrome P450-dependent monooxygenase system. Z. Naturforsch. **45c,** 273–279.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951), Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193,** 265–275.

Müller-Enoch D., Sato N. and Thomas H. (1981), O-Demethylation of scoparone and studies on the scoparone-induced spectral change of cytochrome P450 in rat liver microsomes. Hoppe-Seyler's Z. Physiol. Chem. **362,** 1091–1099.

Müller-Enoch D., Churchill P., Fleischer S. and Guengerich F. P. (1984), Interaction of liver microsomal cytochrome P450 and NADPH-cytochrome P450 reductase in the presence and absence of lipid. J. Biol. Chem. **259,** 8174–8182.

Müller-Enoch D. and Gruler H. (1986), The activation of the cytochrome P450-dependent monooxygenase system by light. Z. Naturforsch. **41c,** 604–612.

Müller-Enoch D. (1993), Localization of cytochrome P450 in membranes: Reconstituted systems. In: Handb. Exp. Pharm. **105** (J. B. Schenkman and H. Greim, eds.). Springer Verlag, Berlin, pp. 71–83.

Nebert D. W., Nelson D. R., Coon M. J., Estabrook R. W., Feyereisen R., Fujii-Kuriyama Y., Gonzalez F. J., Guengerich F. P., Gunsalus J. C., Johnson E. F., Loper J. C., Sato R., Waterman M. R. and Waxman D. J. (1991), The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. DNA Cell Biol. **10,** 1–14.

Omura T. and Sato R. (1964), The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. **239,** 2370–2378.

Ullrich V. and Weber P. (1972), The O-dealkylation of 7-ethoxycoumarin by liver microsomes: A direct fluorometric test. Hoppe-Seyler's Z. Physiol. Chem. **353,** 1171–1177.

Ullrich V. and Schnabel K. H. (1973), Formation and ligand binding of the fluorenyl carbanion by hepatic cytochrome P450. Arch. Biochem. Biophys. **159,** 240–248.

Werck-Reichhart D., Gabriac B., Teutsch H. and Durst F. (1990), Two cytochrome P450 isoforms catalyzing O-deethylation of ethoxycoumarin and ethoxyresorufin in higher plants. Biochem. J. **270,** 729–735.

Werringloer J. and Estabrook R. W. (1973), Evidence for an inhibitory product cytochrome P450 complex generated during benzphetamine metabolism by liver microsomes. Life Sci. **13,** 1319–1330.

Yasukochi Y. and Masters B. S. S. (1976), Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P450) reductase purified by biospecific affinity chromatography. J. Biol. Chem. **251,** 5337–5344.