The Activation of the Cytochrome P-450 Dependent Monooxygenase System by Light

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The cytochrome P-450 dependent monooxygenase system of rat liver microsomes is investigated by light dosimetry (action spectroscopy). The scoparone-O-demethylation activity is enhanced by light and depends on the wavelength of the irradiating light. The relative increase of the activity (about 15%) by the irradiating light (~ 0.5 mW/cm^2) is maximal at a wavelength of 400 nm.

The light induced enhancement of the 7-ethoxycoumarin-O-deethylase activity was measured in reconstituted systems, consisting of one of the two P-450 enzymes (P-450$_{\beta NF-B}$ and P-450$_{PB-B}$) with the NADPH-cytochrome P-450 reductase. The action spectrum of the reconstituted P-450$_{\beta NF-B}$:NADPH-P-450 reductase complex shows a maximum between 420 and 440 nm. The relative increase of the activity induced by light of 420 nm was 7.3% and 9% for the reconstituted systems of P-450$_{\beta NF-B}$ and P-450$_{PB-B}$, respectively.

The results are discussed in analogy to the classic experiments of Warburg with its blocked CO-enzyme-complex. The results can best be explained by the assumption that the light induced enhancement of the enzyme activity is due to an excitement of those intermediate states of the P-450 catalytic cycle (ferric and ferrous state of the heme iron) which are rate limiting.

Introduction

The liver microsomal hydroxylation system catalyzes the oxidative metabolism of numerous hydrophobic endogenous and exogenous compounds [1, 2]. This enzyme system has been termed mixed-function oxidase or monooxygenase [3]. It is generally accepted today that the heme protein cytochrome P-450 [4] acts as terminal oxidase by interacting with the substrate as well as with molecular oxygen [5–7].

The catalytic cycle of a soluble bacterial cytochrome P-450-dependent camphor hydroxylase has been extensively studied by Gunsalus *et al.* [8]. It was shown that the hydroxylation of the substrate involves four essential steps: (1) substrate binding, (2) electron reduction, (3) O_2-binding and (4) second electron uptake.

A good tool to investigate the basic mechanism of the P-450-dependent catalytic cycle at the molecular level is the dosimetry with light. This technique is referred to as analytical action spectroscopy [9]. The advantages of this technique are such that the characteristics of the irradiated light can be varied (*e.g.* light intensity, wavelength, polarisation), and the incubation and the release of the photons in and out of the sample is very fast (determined by the light velocity). It is possible to investigate two basic properties with action spectroscopy: The spectral response and the dynamic response of active groups in the enzymatic systems [9]. Photochemical action spectroscopy was introduced by the classical experiment of Warburg [10] where an inactive ironporphyrin-carbon monoxide complex was reactivated by light. This light-induced reactivation experiment was repeated for the microsomal P-450-CO-complex of the rat liver monooxygenase system by several groups [6, 11, 12].

The classical experiment of Warburg can be interpreted in a physical sense. The light absorption in a molecule such as ironporphyrin, changes its state (ground state $\xrightarrow{h\cdot\nu}$ excited state). The physical as well as the chemical properties of the excited and of the non-excited molecule are different. Therefore one expects that the rate constant or the specific enzyme activity of the excited state is different from that of the non-excited state. In the experiment of

Abbreviations: P-450, liver microsomal cytochrome P-450; naming of individual P-450 enzymes is adapted from Guengerich *et al.* [14]; PB, phenobarbital; βNF, β-naphthoflavone (5,6-benzoflavone).

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Warburg where the enzyme activity was blocked by CO, the enzyme activity of the non-excited state is consequently zero while the enzyme activity of the excited state (absorption of light) is non-zero. In general one might expect for a non-CO-blocked enzyme that the enzyme activity is influenced by the number of absorbed light quantums.

In this study we present evidence to show that light enhances the P-450 dependent monooxygenase enzyme activity in rat liver microsomes as well as in reconstituted purified P-450 enzyme systems ($P\text{-}450_{PB-B}$ or $P\text{-}450_{\beta NF-B}$) with the NADPH-cytochrome P-450 reductase.

Materials and Methods

Materials

Chemicals and biochemicals of the highest purity available were usually obtained from E. Merck (Darmstadt, FR Germany) and Boehringer Mannheim GmbH (Mannheim, FR Germany). Scopoletin (7-hydroxy-6-methoxy-coumarin) and isoscopoletin (6-hydroxy-7-methoxycoumarin) were purchased from Fluka AG (Buchs, Switzerland). These materials were checked for purity by thin-layer chromatography and were used without further purification.

Scoparone (6,7-Dimethoxycoumarin) was prepared by methylation of isoscopoletin with methyliodide, as described previously [13]. 7-Ethoxycoumarin was synthesized as described elsewhere [12]. 7-hydroxycoumarin was purchased from EGA-Chemie KG (Steinheim, FR Germany) and recrystallized from water. All materials used for the purification procedure of the P-450 isozymes and the NADPH-cytochrome P-450 reductase used in this study are described by Guengerich *et al.* [14].

Animals

In all experiments male Sprague-Dawley rats, weighing between 200 and 250 g, which were allowed free access to food and water, 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.

β-Naphthoflavone (βNF) treatment consisted of an intraperitoneal injection of 40 mg/kg body weight in 0.5 ml of corn oil once each day for 3 days prior to sacrifice. Livers were removed and placed in ice-cold 0.25 M sucrose.

Preparation of microsomes

The microsomal fraction was prepared from rat livers washed several times with ice-cold fresh 0.25 M sucrose. The liver was gently homogenized at 0 °C in a Potter homogenizer (3 g liver in 12 ml 0.25 M sucrose). The homogenates were centrifuged at $10000 \times g$ for 20 min (at 4 °C) and the microsomal fraction was obtained by centrifuging the $10000 \times g$ supernatant fluid at $105000 \times g$ for 60 min (4 °C). The pellet obtained was washed with isotonic KCl, again sedimented at $105000 \times g$ for 60 min and resuspended in 0.05 M Tris buffer of pH 7.6 to give a final protein concentration of 10 mg/ml. All experiments were performed with microsomes on the day of their preparation. Until they were used, microsomes were kept in closed vessels at 0 °C in an ice bath. Protein was determined by the method of Lowry *et al.* [15] with bovine serum albumin as a standard.

Purification of cytochromes P-450 and of the NADPH-Cytochrome P-450 reductase

Liver $P\text{-}450_{PB-B}$ (apparent monomeric M_r = 50,000) and NADPH-cytochrome P-450 reductase (M_r = 74,000) were purified to electrophoretic homogeneity from PB-treated rats using procedures described by Guengerich *et al.* [14]. The same reference describes the purification of the $P\text{-}450_{\beta NF-B}$ (M_r = 54,000) from βNF-treated rats. The NADPH-P-450 reductase had a specific activity of 42 μmol cytochrome c reduced per min and mg of protein. The $P\text{-}450_{PB-B}$ and $P\text{-}450_{\beta NF-B}$ had a specific content of 18.5 and 17.3 nmol of P-450 heme per mg of protein, respectively.

Spectrophotometry

The cytochrome P-450 content was determined by recording the CO-reduced minus reduced difference spectra according to Omura and Sato [4] using an extinction coefficient of 91 $mM^{-1} \times cm^{-1}$. The NADPH-cytochrome P-450 reductase activity was determined according to Yasukochi and Masters [16] and expressed as NADPH-cytochrome *c* reductase activity at 30 °C in 0.3 M potassium phosphate buffer pH 7.7. Difference spectra and absolute spectra of the purified P-450 enzymes and the reductase were recorded from 370–500 nm using a Varian Cary spectrophotometer model 219 Varian GmbH (Darmstadt, FR Germany).

Reconstitution of the P-450 enzymes with the NADPH-cytochrome P-450 reductase

One of the two purified P-450$_{PB-B}$ or P-450$_{\beta NF-B}$ enzymes was mixed with the NADPH-P-450 reductase in small test tubes (final volume 50 µl) by adding the P-450 enzymes to the reductase in a 1:2 molar ratio at high concentrations (3.7 and 7.2 µM) as described by Müller-Enoch *et al.* [17].

Complexes of P-450$_{PB-B}$ or P-450$_{\beta NF-B}$ with the NADPH-P-450 reductase are formed after 60 min incubation at 23 °C. Therefore this system is named preformed or reconstituted P-450: NADPH-P-450 reductase complex.

Analytical action spectroscopy

a) Liver microsomes

(i) Irradiation of the microsomes

The test system for the scoparone demethylation reaction contained in a total volume of 0.5 ml: 50 µl of the substrate scoparone (10^{-3} M in 0.1 Tris-chloride buffer, pH:7.6), 100 µl of microsomal protein (10 mg/ml), 10 µl $MgCl_2$ (10^{-1} M) and 300 µl of 0.1 M Tris-chloride buffer, pH = 7.6 in a quartz cuvette.

The cuvette was kept in the sample holder for two min, while the temperature was maintained at 26 °C using a water bath (Colora WK7). The reaction was initiated by adding and mixing 10 µl NADPH (10^{-2} M) to the test system. The irradiation of this microsomal suspension was done in a fluorescence spectrometer (Schoeffel RRS 1000). The optical slits were adjusted in such a way that the photochemical degradation of the product scopoletin (see results) is minimized. The light intensity at the sample was ~ 0.5 mW/cm^2. The wavelength distribution of the beam had a half width of about 15 nm. The irradiation of the microsomal suspension was done in such a way that the light passes a narrow channel (4 mm × 12 mm window and 10 mm length) with an aluminium foil placed behind the cuvette. The advantage of this instrumental arrangement is that the wavelength of the irradiation can be changed continuously.

(ii) Fluorometric determination of enzyme activity

The microsomal O-demethylation activity can directly be determined by recording the fluorescence of the product scopoletin, since the substrate scoparone and the product scopoletin exhibit different excitation and emission behavior [18]. The spectrum of the substrate scoparone has an excitation maximum at 355 nm, and an emission maximum at 440 nm. The absorption maximum of the product scopoletin is at 398 nm and the maximum of fluorescence at 460 nm. For the determination of enzyme activity an excitation wavelength of 400 nm was chosen since for this wavelength the observed fluorescence of the substrate is nearly zero but maximal for the product. The fluorescence intensity of the product scopoletin is a direct measure of the O-demethylation activity as shown in the insert of Fig. 1 ($\lambda_E = 400$ nm, $\lambda_F = 460$ nm).

The fluorescence spectra ($\lambda_E = 400$ nm; $\lambda_F = 420-550$ nm) for the substrate scoparone in the absence of NADPH is shown in Fig. 1 (left side) indicated by $I_{inactive}$ (λ_F). From this spectrum, the intensity at $\lambda_F = 460$ nm is determined as $I_{inactive}$ (460). The fluorescence spectra ($\lambda_E = 400$ nm, $\lambda_F = 420-550$ nm) after a reaction time of 5 min and 20 sec in the presence of NADPH is also shown in Fig. 1 (left side). This spectrum is termed as I°_{active} (λ_F), where I° indicates that the sample was not irradiated by light during the 5 min period of product formation. From this spectrum, the intensity at $\lambda_F = 460$ nm is determined as I°_{active} (460). The spectra I°_{active} (λ_F) is shown for two different measurements without irradiation of the sample cuvette during the 5 min period. The difference of these fluorescence intensities I°_{active} (460) − $I_{inactive}$ (460), is directly proportional to the concentration of the product scopoletin. This difference was measured several times during the course of the experiment because it represents the enzyme activity in the dark and therefore has to be used as the standard (see dotted line in the upper part of Fig. 1). The numerous curves in the upper part of Fig. 1 represent a part of the fluorescence spectra after irradiation of the sample with a constant wavelength λ_{irr} during the 5 min incubation time. The wavelength of the irradiation, which is indicated by the scale on top of Fig. 1, was varied for each experiment (in duplicate). For the quantification of the light induced product formation we have to determine the fluorescence intensity at the wavelength $\lambda_F = 460$ nm from the fluorescence curves (Fig. 1). It will be described by $I^{\lambda irr}_{active}$ (460) where λ_{irr} denotes the wavelength of the irradiated light in the 5 min period. The difference of the fluorescence intensities, $I^{\lambda irr}_{active}$ (460) − $I_{inactive}$ (460), is directly proportional to the

concentration of the product scopoletin. The following irradiation factor $f(\lambda_{irr})$ was calculated according to

$$f(\lambda_{irr}) = \frac{I^{\lambda irr}_{active}(460) - I_{inactive}(460)}{I^{\circ}_{active}(460) - I_{inactive}(460)}.$$

The enhancement of the product formation produced by light is measured by $f(\lambda_{irr}) > 1$.

There is a difficulty that the irradiation of the incubation mixture provokes a degradation of the product scopoletin. Therefore, in order to obtain the real product formation from scoparone the fluorescence intensity $I^{\lambda irr}_{active}(460)$ has to be corrected for degradation of scopoletin. The following procedure was applied: (i) the fluorescence intensity $I^{\circ}_{active}(460) - I_{inactive}(460)$, was calibrated with a known concentration of scopoletin according to the procedure of Müller-Enoch *et al.* [18]. The concentration of the product averaged over the 5 min incubation time was 0.3 μM. (ii) The decrease due to the photochemical degradation of a 0.3 μM solution of scopoletin in 0.1 M Tris-chloride buffer of pH 7.6 at 26 °C at an irradiation wavelength of 350, 375, 400, 420, or 450 nm was 1.9%, 3.7%, 4.2%, 1.7% and 0%, respectively. (iii) The correction factors for the photochemical degradation of the product scopoletin can be determined for the different irradiation wavelengths: 1.019 (350 nm), 1.037 (375 nm), 1.042 (400 nm), 1.017 (420 nm), 1.0 (450 nm).

b) Irradiation of the reconstituted P-450: NADPH-P-450 reductase complexes and the determination of the 7-ethoxycoumarin O-deethylase activity

The preformed P-450: NADPH-reductase complex was diluted with 0.1 M Tris-HCl buffer (pH 7.6) containing 20% glycerol to give a final concentration of P-450 and NADPH-P-450 reductase of 0.95 and 1.9 μM respectively. Aliquots (5 μl) were added to a cuvette containing in a total volume of 100 μl 0.1 M Tris-Chloride buffer (pH 7.6) with 20% glycerol (v/v): 40 nmol 7-ethoxycoumarin, 0.5 μmol $MgCl_2$ and 25 nmol NADPH. In each experiment the initial rate (1–4 min) of the 7-hydroxycomarin formation was followed after starting the reaction by adding the aliquots of the preformed complexes. Exactly at one minute after starting the reaction, the 3 min irradiation period using light with different wavelengths ($\lambda_{irr} = 380$, 400, 420, 440, and 460 nm) was begun and 4 min and 10 seconds after starting the reaction (the zero time) the recording of the 7-hydroxycoumarin was continued. Each assay was measured and calibrated by adding an aliquot of 7-hydroxycoumarin as described by Ullrich and Weber [12]. The following factor $f(\lambda_{irr})$ was calculated according to:

$$f(\lambda_{irr}) = \frac{\text{spec. activity of the irradiated sample}}{\text{spec. activity of the non-irradiated sample}}.$$

The product 7-hydroxycoumarin shows no photochemical degradation for light of the wavelengths used.

The absorption maxima of 7-ethoxycoumarin and 7-hydroxycoumarin are 330 nm and 365 nm, respectively. The purified P-450 enzymes have an absorption maximum at 417 nm, and the reductase has two broad maxima at 380 nm and 455 nm.

Under the conditions used and under the assumption that all the light is absorbed in the aqueous sample and if the sample would be isolated from the heat bath one calculates for the three minutes radiation time an increase of the sample temperature of 0.1 °C.

Results

Irradiation of liver microsomes

The activity of the P-450 dependent microsomal monooxygenase system can be determined by the direct fluorescence method using scoparone as substrate [18]. We found that this activity can be enhanced by irradiating with light as can be seen directly from the fluorescence spectra (uncorrected) in the upper part of Fig. 1. The dotted line represents the activity of the non-irradiated samples. At each irradiation wavelengths used, the fluorescence intensity of the irradiated sample with its maximum at 460 nm is obviously higher than the dotted line and therefore an indication of an enhanced product formation. This light induced enhancement of the product formation can be quantitated by calculating the factor $f(\lambda_{irr})$. The irradiation of the incubation mixture has not only an effect on the enzymatic activity but also on the degradation of the product scopoletin. Therefore, the measured data was corrected for the degradation of scopoletin (see experimental procedure). The degradation is maximum at a irradiation wavelength of 400 nm. The factor for this irradiation wavelength is 1.09 and 1.14 before and after correction, respectively. This means that the increase of the microsomal enzym activity measured as the product

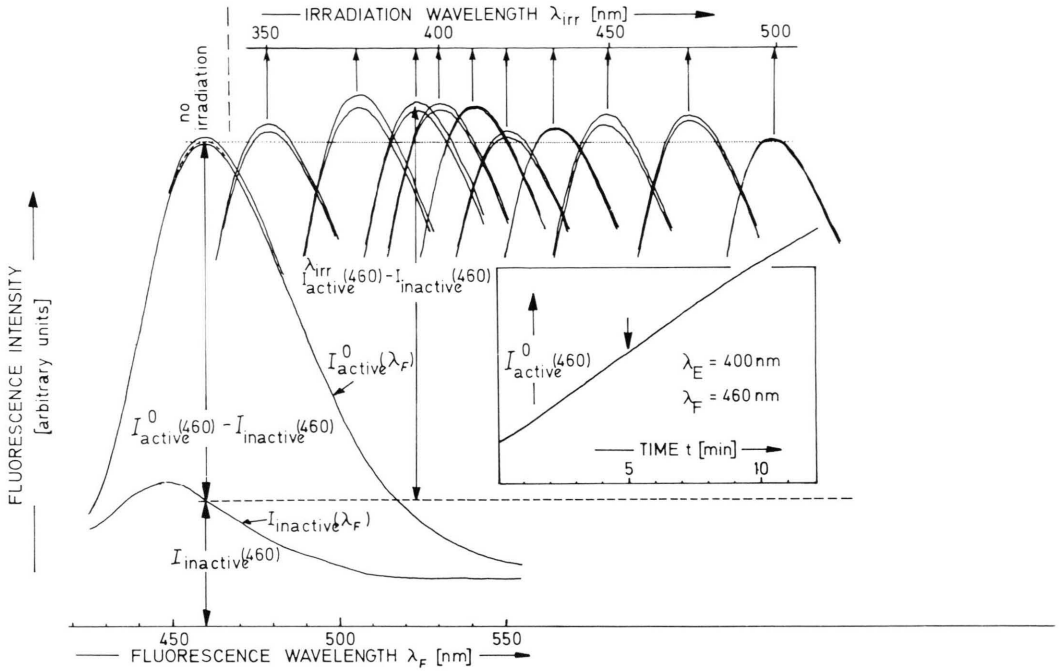

Fig. 1. Formation of scopoletin from scoparone by rat liver microsomes. The reaction mixture contained in a total volume of 0.5 ml: 50 µl of 10^{-3} M scoparone in 0.1 M Tris-chloride buffer (pH = 7.6), 100 µl of microsomal protein (10 mg/ml), 10 µl of 10^{-1} M $MgCl_2$ and 300 µl of 0.1 M Tris-chloride buffer (pH = 7.6). After 2 min preincubation at 26 °C, the reaction was initiated by adding 10 µl of 10^{-2} M NADPH to the sample cuvette. At the same time, the 5 min period of irradiation of the indicated wavelengths was begun. Five min and 20 sec after starting the reaction, the recording of the scopoletin fluorescence spectrum (excitation 400 nm, recording scan, 420–550 nm) was started. Each experiment was performed in duplicate. The spectrum $I_{inactive}$ (λ_F) shows the fluorescence without NADPH under the above mentioned conditions. The upper left part of the figure represents the fluorescence spectrum ($\lambda_E = 400$ nm, $\lambda_F = 420-550$ nm) after the 5 min and 20 sec reaction time in the dark, indicated by I^0_{active} (λ_F). The inset shows that the production of scopoletin ($\lambda_E = 400$ nm, $\lambda_F = 460$ nm) remains linear within the first 10 min. The dotted line in the upper part of the figure represents the fluorescence intensity ($\lambda_E = 400$ nm, $\lambda_F = 460$ nm) without irradiation. The fluorescence spectra (shown as duplicates) taken after the 5 min irradiation are drawn in the upper part of the figure. The spectra are uncorrected and the irradiation wavelength (λ_{irr}) is indicated on top of the figure. The fluorescence intensity above the dotted line represents the enhanced product formation after irradiation.

formation scopoletin is 9% (uncorrected data) and 14% (corrected data) for an irradiation with light of 400 nm.

Fig. 2 is a collection of the results (corrected data) of several sequences of measurements with microsomal preparations from different animals. We find that the microsomal monooxygenase activity is wavelength-dependent, with a broad maximum at about 400 nm, and a weak one at about 450 nm.

The microsomal monooxygenase activity was measured for different intensities: The enzyme activity is enhanced by a factor of 1.15 ± 0.03 and 1.06 ± 0.03 (corrected values) for the light intensity 0.5 and 0.25 mW/cm^2, respectively, at 400 nm.

Irradiation of the preformed complex of NADPH-P-450 reductase with P-450$_{\beta NF-B}$ or P-450$_{PB-B}$

The enzyme activities of the preformed complexes were determined fluorimetrically with 7-ethoxycoumarin as a substrate. The fluorescence intensity of the product 7-hydroxycoumarin is shown as a function of time in Fig. 3. The specific activities determined from recordings like Fig. 3 change with the wavelength of the irradiation. The results are shown in Fig. 4. The reconstituted system with P-450$_{\beta NF-B}$ (specific activity without irradiation: 32 nmol 7-hydroxycoumarin × min^{-1} × nmol P-450$_{\beta NF-B}{}^{-1}$) is activated by light in the range of 400 to 460 nm, with a maximum between 420 and 440 nm. The reconsti-

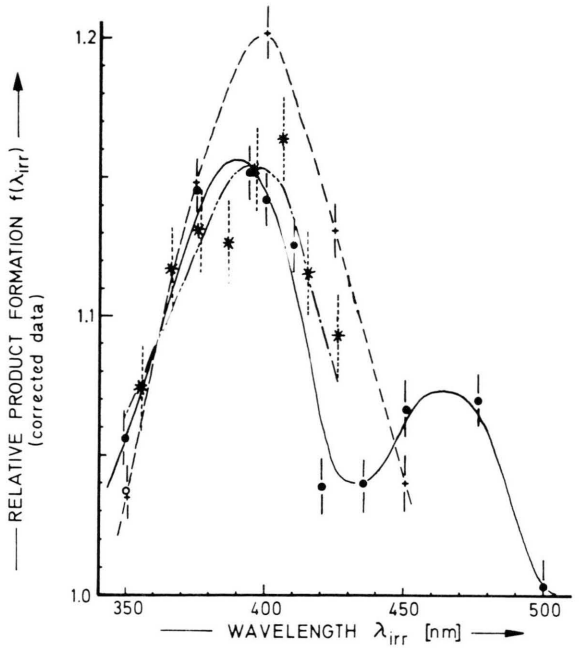

Fig. 2. Photochemical action spectrum of the O-demethylation of scoparone by rat liver microsomes. The experimental procedure has been described in Fig. 1. The enzymatic activity is corrected for the photochemical degradation of the product scopoletin. The plot represents the enhancement of the product formation as a function of the irradiating wavelength of three different (●), (+) and (✳) microsomal fractions from rats. The bars indicate the range of the measurements.

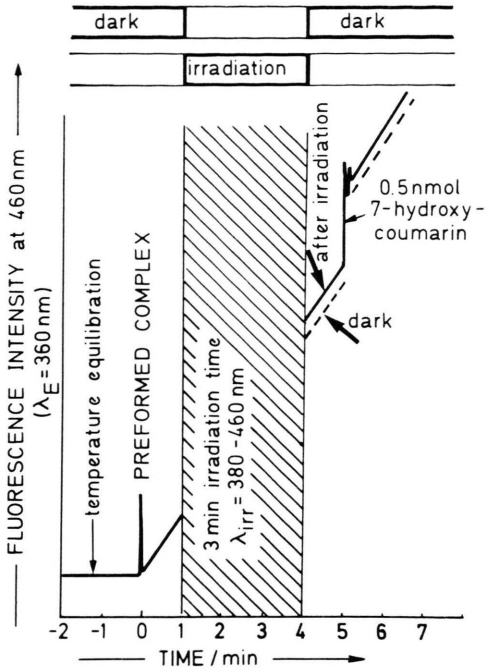

Fig. 3. Estimation of the specific activities of the 7-ethoxycoumarin O-deethylase of reconstituted systems of P-$450_{\beta NF-B}$ and NADPH-P-450 reductase with and without light. The sample cuvette contained in a total volume of 100 µl of 0.1 M Tris-chloride buffer (pH = 7.6): 20% glycerol (v/v), 40 nmol 7-ethoxycoumarin, 0.5 µ mol $MgCl_2$ and 25 nmol NADPH. After 2 min preincubation at 26 °C, the reaction was initiated by adding aliquots (5 µl) of the reconstituted enzyme complexes. The concentrations in the cuvette were 0.047 µM for the P-$450_{\beta NF-B}$ and 0.095 µM for the NADPH-P-450 reductase. After plunging, the fluorescence of the product 7-hydroxycoumarin ($\lambda_E = 360$ nm, $\lambda_F = 460$ nm) was followed. Exactly at 1 min after starting the reaction, the 3 min irradiation period using light with different wavelengths in the range of 380–460 nm (see Fig. 4) was begun and 4 min and 10 sec after starting the reaction the recording of the 7-hydroxycoumarin was continued. Each assay was calibrated with an aliquot of 7-hydroxycoumarin. The thick line represents the fluorescence after irradiation light and the dotted line without irradiation.

tuted system with P-450_{PB-B} (spec. activity without irradiation: 12 nmol 7-hydroxycoumarin × min^{-1} × nmol P-$450_{PB-B}{}^{-1}$) was also measured at 420 nm where a 9% increase was observed at the same wavelength (data not shown).

Spectral properties of the P-$450_{\beta NF-B}$, the NADPH-P-450 reductase and the preformed complex

The absolute spectrum of the oxidized hemoprotein P-$450_{\beta NF-B}$ has a known absorbance maximum at 417 nm [14, 19]. After reduction by sodium dithionite the peak shifts downward to 411 nm accompanied by a decrease in the extinction coefficient and the appearance of a broad shoulder between 430 and 480 nm and a dramatic absorption below 395 nm [19]. The difference spectrum of the oxidized versus the sodium dithionite reduced cytochrome P-$450_{\beta NF-B}$ shows two maxima at 444 nm and 460 nm and a minimum at 417 nm.

The absolute spectrum of the preformed complex is shown in Fig. 5. The absorbance peak at 417 nm belongs to the P-$450_{\beta NF-B}$ component. The broad shoulder around 455 nm and the absorption at 380 nm result from the oxidized NADPH-P-450 reductase component. Addition of NADPH changes the absorption spectrum totally. The peak at 417 nm shifts to 415 nm, the broad shoulder at 455 nm decreases and a large absorption below 395 nm occurs. Two minutes after addition of the substrate 7-ethoxy-

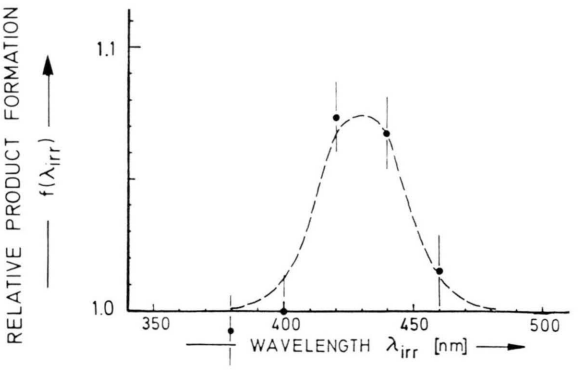

Fig. 4. Photochemical action spectrum of the O-deethylation of 7-ethoxycoumarin by the preformed complex of P-$450_{\beta NF-B}$ and NADPH-P-450 reductase. The specific activity of the 7-ethoxycoumarin O-deethylation is determined as described in Fig. 3. P-$450_{\beta NF-B}$ (●) at 26 °C had a specific activity (no irradiation) of 32 nmol 7-hydroxycoumarin × min^{-1} × nmol $P\text{-}450^{-1}$. The light intensity used for the irradiation was ~ 0.5 mW/cm^2. The values shown are the average of three different measurements. The bars indicate the range of the measurements.

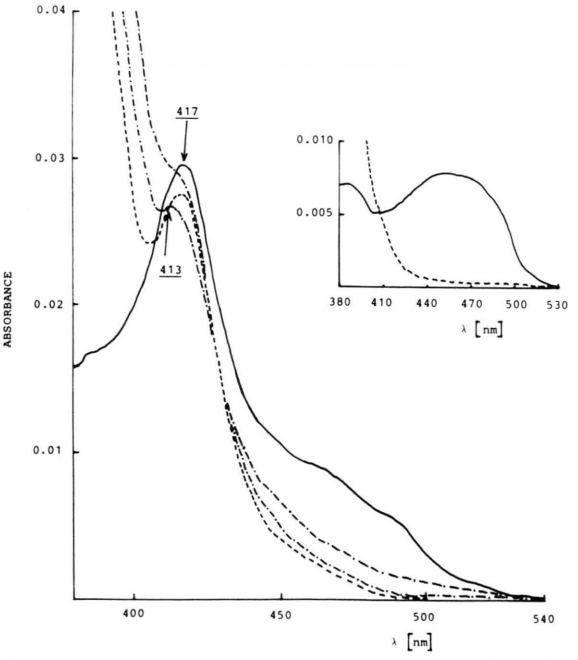

Fig. 5. Absolute spectra of the preformed complex of P-$450_{\beta NF-B}$:NADPH-P-450 reductase. The absolute spectrum (——) was recorded at P-$450_{\beta NF-B}$ and reductase concentrations of 0.25 and 0.50 μM respectively in 0.8 ml 0.1 M Tris/HCl-buffer (pH 7.6) containing 20% glycerol. The dashed line represents the absolute spectrum after adding 100 μM NADPH to the cuvette. The two dashed dotted lines were recorded after 2 min (−●−) and after 5 min (●−−●) following the addition of 50 μM 7-ethoxycoumarin to the reaction mixture. The inset shows the absolute spectrum of the NADPH-P-450 reductase (0.50 μM). The dashed line shows the result after reduction with 100 μM NADPH.

coumarin the peak originally located at 417 nm shows a further shift to 413 nm indicating a further reduction of the ferric form to the ferrous form. This is also indicated by a slight increase of the small broad shoulder between 440–480 nm which increases with time.

Discussion

A deactivation of the microsomal benzo (a) pyrene hydroxylase activity by light in the presence of methylene blue has been described by Rahimtula *et al.* [20]. In contrast to these results, we find a light-induced activation of the scoparone O-demethylation in rat liver microsomes which is slightly enhanced by irradiation in a range from 360 to 480 nm. Franklin, Estabrook and Ullrich also found a similar effect. Their results were presented as unpublished experiments in Fig. 6 of an article by Ullrich and Schnabel [21]. Pohl and Fouts [22] found a light-induced enhancement of the activity of the *in vivo* induced 7-ethoxycoumarin deethylation measured in pieces of whole skin from Hrs/J strain hairless mice after exposure to "sunlamp" radiation (280–750 nm).

Since in rat liver microsomes numerous forms and states of cytochrome P-450 are present with different spectral properties, it is impossible to correlate the photochemical action spectrum of scoparone by rat liver microsomes with one of its functional states. Therefore we purified two of the P-450 enzymes, namely P-$450_{\beta NF-B}$ and P-450_{PB-B}, to electrophoretic homogeneity as well as the NADPH-cytochrome P-450 reductase, and studied the light effect with these two reconstituted pure monooxygenase systems. The purified reconstituted system has also the advantage that it is not turbid like the microsomal suspension. Hence, inner filter effects which might originate from light scattering are excluded to a large extent. Instead of scoparone as substrate where the product scopoletin was slightly decomposed by light, we have employed 7-ethoxycoumarin where the product 7-hydroxycoumarin shows no product decomposition by the light used.

The action spectrum of the reconstituted complex shows a maximum between 420 and 440 nm (Fig. 4).

This spectrum has to be compared with the absorption bands of the species which are involved in the reaction. The absorption peak of the ferric P-450$_{\beta NF-B}$ at 417 nm as well as the ferrous state at (411 nm, Ryan *et al.* [19], see also (Fig. 5) could explain the left part of the maximum in the action spectrum. The substrate (7-ethoxycoumarin) as well as the product (7-hydroxycoumarin) cannot explain the action spectrum because these molecules have an absorption at 330 nm and 365 nm, respectively. The reductase has a very broad absorption between 440–470 nm (insert of Fig. 5). This absorption is still significant at about 500 nm. However, the action spectrum (Fig. 4) shows no activation for wavelengths larger than 460 nm. Therefore, the best correlation between the action spectrum and the absorption spectrum holds for the P-450 component.

One rate limiting step in the catalytical cycle for cytochrome P-450 dependent hydroxylation reactions is the product dissociation, where the hydroxyl group of the product is coordinated to the iron by the hydroxyl-oxygen immediately after its formation, which may have an inhibitory effect for a new substrate binding. Therefore, it is possible, that the dissociation of the enzyme-product complex is facilitated by light at about 420 nm, the absorption maximum of low spin hexacoordinate iron-alcohol complexes [23], thereby enhancing the binding of a new substrate molecule.

Another effect of the product binding is that the dioxygen binding to the ferrous iron cannot occur, because the oxygen atom of the hydroxyl group acts as an electron donor and competes with the molecular oxygen for the catalytic site (ferrous iron). A broadening of the shoulder at about 430–470 nm is seen from the steady state experiment with the reconstituted enzyme system – see in Fig. 5 the two dashed dotted lines (–●–) and (●––●) – which shows the reduction of the ferric cytochrome P-450$_{\beta NF-B}$ (417 nm) to the ferrous cytochrome (413 nm) accompanied by an enhancement of the shoulder at 430–500 nm with the reaction time (2 min, and 5 min). Numerous nucleophilic ligands are reported to react with the reduced state of cytochromes by producing complexes with the ferrous iron, having absorption maxima at about 450 nm, and competing with the dioxygen for the sixth ligation position [21, 24–30]. Ullrich and Schnabel [21] have shown the light reversibility of the piperonyl butoxide-inhibited demethylation of ethylmorphine (photo-activation of a non-productive complex other than P-450-CO).

If a fraction of the cytochrome is blocked, as in the case of CO or hydroxylated product ligation to the ferrous iron, then that population of the cytochrome is eliminated from the oxygen activation process. Therefore, it is highly likely that upon irradiation with light, a photodissociation of the ferrous iron-product complex occurs and results in a reversal of that inhibition.

Other possible rate limiting steps in the proposed catalytic cycle [7, 31, 32] are the transfer of the second electron to the ferrous dioxygen complex and the generation of the "active oxygen" as has been reviewed by White and Coon [32]. The proposed ferrous dioxygen complexes which show absorption maxima at about 440 nm were first reported by Estabrook *et al.* [31]. Rösen and Stier [33] and Guengerich *et al.* [34] gave further evidence by flash photolysis experiments and stopped flow spectrophotometry, respectively, for the occurrence of oxyferro complexes which have absorption maxima at about 423 and 445 nm. The decomposition of an oxyferro complex having a broad maximum centered at 444 nm is referred to as ratelimiting [34]. Therefore, it may be possible that the irradiation with light at about 440 nm has an activating effect on those intermediates of the catalytic cycle and may also explain the right side of the broad maximum of the action spectrum (Fig. 4).

The light induced enhancement of the enzyme activity can only act in the intermediate states which are rate limiting for the catalytic reaction. In the case of the CO blocked enzyme activity, the light excites this state resulting in a photodissociation of the enzyme-CO-complex. Our results were discussed in this framework. Photodissociation of the enzyme-product-complex (ferric and ferrous iron state) as well as of the enzyme-water-complex (ferric state) [32] are possible as rate limiting molecular models. A further rate limiting step is the ferric dioxygen complex with the release of a water molecule. Such intermediate states migth also contribute to the action spectrum.

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