Light-Induced Activation and Synchronization of the Cytochrome P-450 Dependent Monooxygenase System

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The light-induced enhancement of the 7-ethoxycoumarin-O-deethylase activity was measured in a reconstituted system, consisting of the enzyme P-450$_{PB-B}$ and the NADPH-cytochrome P-450 reductase. The relative increase of the activity was about 15%. It is shown that the product release process is accelerated by light. The phases of the catalytic cycle of 2×10^{12} protein complexes were locked by periodic application of light pulses (0.1 s duration, 1.32 s repetition time, and 390–470 nm, 0.27 joule/nmol P-450). More than 80% of the active reconstituted enzyme complexes (= "molecular machines") worked in phase after a few light pulses. The phase relation continued even after switching off the light pulses. The catalytic cycle time was 1.54 s, giving a turnover number of 39 min^{-1}. The turnover number, as determined from the enzyme activity under optimum conditions, was 39 min^{-1}. Due to the dissociation constant of the P-450$_{PB-B}$: NADPH-P-450 reductase complexes [3] only 24% of the proteins were in the active (= working) state under the conditions used. The lifetime of this complexes is larger than 6 s since more than 4 cycles of the free running enzyme can be observed. This is the first report, that all catalytic active complexes in the test tube can be synchronized by an external light source, if the right repetition time of the pulses is chosen, so that all these "molecular machines" work in phase.

Introduction

The enzyme activation of heme-proteins with light started with the classical experiment of Warburg [1] where the enzyme is blocked by an inhibitor as for example carbon monoxide. The light absorption in a molecule such as ironporphyrin × CO, leads to a dissociation and the inhibitor CO is released. Then the catalytic activity of the enzyme is recovered, ready for binding to a substrate molecule. The light activation is, simply spoken, the enlargement of the number of working enzymes. A new type of enzyme activity enhancement with light was discovered by us [2] for cytochrome P-450 dependent activities without CO inhibition. We showed that the light-induced enhancement of the enzyme activity is due to an excitement of those intermediate states of the P-450 catalytic cycle which are rate limiting. In general one expects that the rate constant of an excited state is different from that of a non-excited state. In this experiment we confirmed our results, given in ref. [2] with a newly designed apparatus and we were able to show that the light-induced enhancement can be up to 15% for an isolated and reconstituted cytochrome P-450 reductase-complex.

The overall characterization of the enzyme activity of a given isolated enzyme and substrate is the turnover number. This number is obtained by extrapolating the measured enzyme activity in the case of optimum conditions [3]. In this paper we will show that the turnover number can be determined in a single experiment without optimum conditions since we were able to synchronize a large percentage of the actively working enzymes in a test tube and the time required for a single catalytic cycle can thus be measured directly. This time is the reciprocal of the turnover number. The used technique to slave the catalytically working enzymes consisted in applying periodic light flashes which induced periodic in-phase working enzymes.

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The following procedures have been suggested to investigate the basic mechanism of the P-450-dependent catalytic cycle [4] at the molecular level: (i) the flash photolysis experiments of the carbon monoxide inhibited enzyme system [5–9], (ii) the stopped-flow experiments [9, 10] where the cycle is started by mixing the reaction components, and (iii) the low temperature experiments where the first reduction step is inhibited [11]. The first two types of experiments can be regarded as relaxation experiments since the catalytic cycle is started at a defined instant time. The different intermediate states of the catalytic cycle can be optically monitored by their absorption spectra or difference spectra or by other techniques. The measured relaxation curves can be fitted by the rate constants between the different intermediate states. However, the main problem is, that the relaxation curves yield by only a few parameters but the complex catalytic cycle has to be described by a large number of rate constants or other fitting parameters. To solve this problem, experiments which provide amount of information have to be performed. A logical next step is the extension of the relaxation experiments to the periodical in-phase working enzymes where much more information can be gathered.

Materials and Methods

Materials

Chemicals and biochemicals of the highest purity available were usually obtained from E. Merck (Darmstadt, F.R.G.) and Boehringer Mannheim GmbH (Mannheim, F.R.G.). 7-Ethoxycoumarin was synthesized as described elsewhere [7]. 7-Hydroxycoumarin was purchased from EGA-Chemie KG (Steinheim, F.R.G.) and recrystallized from water. All materials used for the purification procedure of P-450 reductase used in this study are described by Guengerich *et al.* [12].

Purification of cytochrome P-450$_{PB\text{-}B}$ and of the NADPH-cytochrome P-450 reductase

Liver P-450$_{PB\text{-}B}$ (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.* [12]. The NADPH P-450 reductase had a specific activity of 42 μmol cytochrome *c* reduced per min and mg of protein. The P-450$_{PB\text{-}B}$ had a specific content of 15 nmol of P-450 heme per mg of protein. Protein was determined by the method of Lowry *et al.* [13] with bovine serum albumin as a standard.

The cytochrome P-450 content was determined by recording the CO-reduced minus reduced difference spectra according to Omura and Sato [14] using an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$. The NADPH-cytochrome P-450 reductase activity was determined according to Yasukochi and Masters [15] 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 cytochrome P-450$_{PB\text{-}B}$ enzyme and the reductase were recorded from 370–500 nm using a Varian Cary spectrometer model 219 (Varian GmbH, Darmstadt, F.R.G.).

Reconstitution of the P-450$_{PB\text{-}B}$ enzyme with the NADPH-cytochrome P-450 reductase

The purified P-450$_{PB\text{-}B}$ enzyme was mixed with the NADPH-P-450 reductase in small test tubes (finale volume 50 μl) by adding the P-450 enzyme to the reductase in a 1:2 molar ratio at high concentrations (7.5 and 15 μM) as described by Müller-Enoch *et al.* [3]. Complexes of P-450$_{PB\text{-}B}$ 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.

Irradiation of the reconstituted P-450$_{PB\text{-}B}$: NADPH-P-450 reductase complex and determination of the 7-ethoxycoumarin-O-deethylase activity

7-Ethoxycoumarin-O-deethylase activity were assayed using the continuous fluorometric assay [7] and showed a specific activity of 9.2 nmol 7-hydroxycoumarin per min and nmol P-450$_{PB\text{-}B}$ under the conditions used at 32 °C. The turnover number of this preformed complex is the specific activity under optimum conditions described by Müller-Enoch *et al.* [3] and is referred to as having a value of 39 min^{-1}. This is the production of 39 nmol 7-hydroxycoumarin per nmol P-450$_{PB\text{-}B}$ and per min at 32 °C. The reciprocal of the turnover number gives the time required for a single catalytic

cycle (= cycle time τ). Thus for cytochrome P-450$_{PB-B}$, the catalytic cycle time is 1.54 s.

The light-induced enhancement of the 7-ethoxycoumarin-O-deethylase activity was performed with preformed P-450:NADPH-reductase complexes, diluted by 0.1 M Tris-HCl buffer (pH 7.6) containing 20% glycerol to yield a final concentration of P-450 and NADPH-P-450 reductase of 1.875 and 3.75 μM, respectively. Aliquots of this complex (8 μl ≙ 15 pmol P-450$_{PB-B}$) were added to a cuvette which contained in a total volume of 130 μl: 0.1 M Tris-chloride buffer (pH 7.6), 20% glycerol (v/v), 0.1 mM 7-ethoxycoumarin, 3.3 mM $MgCl_2$ and 0.115 mM NADPH.

The irradiation of the reaction mixtures was performed by a special computer-aided fluorescence spectrometer as shown in Fig. 1. One light source (XBO 150 W/1, Osram München, F.R.G.) was adjusted to the excitation wavelength of 7-hydroxycoumarin (interference filter, type UV-AL, Schott, Mainz, F.R.G.: 364,6 ± 8.0 nm) and the detection unit was oriented perpendicular (interference filter type AL, Schott, Mainz: 460.2 ± 10.5 nm; photomultiplier (EMI type 9789 QA)). The second light source (XBO 150 W/1), perpendicular to the detection system, was adjusted to the action spectra [2] of the reconstituted P-450:NADPH-P-450 reductase complexes (390–470 nm, filter KV 389 and BG 37, Schott, Mainz, F.R.G.). The irradiation time as well as the repetition time, *T*, could be varied over a wide range by switching a chopper, controlled by a computer. The light intensity at the position of the sample was measured with a radiometer/photometer (Model 450-1 in connection with multiprobe head 550-2, EG&G Electronic-optics, Salem, Mass.).

Results and Discussion

Light-induced enhanced enzyme activity

The activity of P-450 with and without irradiation is shown in Fig. 2. The fluorescence intensity which is proportional to the content of the formed

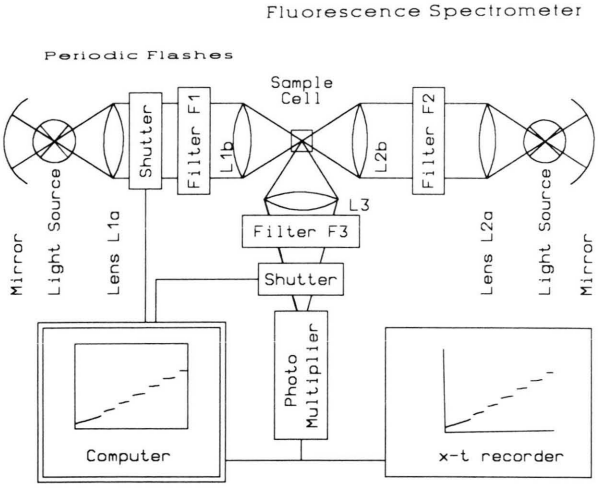

Fig. 1. Schematic representation of the used apparatus: Computerized fluorescence spectrometer with an additional independent light source. On the right part a fluorescence spectrometer is shown. The excitation (excitation wavelength (filter F 2) 364.6 ± 8.0 nm) is perpendicular the detection (fluorescence wavelength (filter F 3) 460.2 ± 10.5 nm). The second light source on the left side generates by means of a shutter periodic light flashes (filter F 1: 430 ± 40 nm). The fluorescence signal is interrupt for 0.3 s; the duration of the flash was 0.1 s. The repetition time and the duration of the flash was controlled by a computer program.

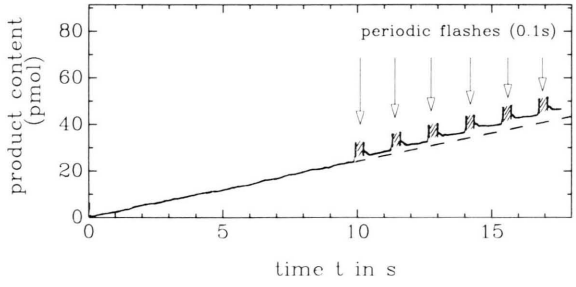

Fig. 2. Effect of periodically (1.32 s) applied short light pulses (0.1 s, 390–470 nm) on the product formation of the 7-ethoxycoumarin-O-deethylase activity produced by a reconstituted P-450$_{PB-B}$:NADPH-P-450 reductase complex. The incubation mixture contained in a total volume of 130 μl: P-450$_{PB-B}$ (0.115 μM), reductase (0.231 μM), 0.1 mM 7-ethoxycoumarin, 0.115 mM NADPH, and 0.33 mM $MgCl_2$. During the first 10 s the sample was not irradiated by the additional light source. The dashed line is the extrapolated product increase without the additional irradiation. The additional light pulses cause an increase of the product formation since the measured fluorescence intensity is always above the prediction (dashed line). The light pulses also cause a surprising effect – the product content in the cuvette increases in a step-like manner indicating a light-induced product release.

product (7-hydroxycoumarin), is shown as a function of time. The reaction was started by mixing an aliquot of the preformed complex to the reaction mixture (7-ethoxycoumarin, NADPH, and $MgCl_2$ in Tris buffer) in the thermostated cuvette. After 10 s the recording of the reaction was started. The product formation was measured fluorescence spectroscopically. (The used irradiation for the product detection had no influence on the enzyme activity. The periodic flash irradiation of a 10 µmol solution of the product had no effect on the fluorescence intensity.) The straight line behaviour between 0 and 10 s (Fig. 2) indicates that the enzyme activity is constant and we proofed (graphs not shown) that this linear behaviour continuous up to 5 min under such conditions. The extrapolation of the expected increase in fluorescence intensity is shown as a dashed line. From 10 to 18 s the sample was irradiated with light pulses (wavelength 390 to 470 nm, pulse duration 0.1 s, repetition time 1.32 s, and 0.27 J/nmol P-450) which had an effect on the specific enzyme activity. No measurements were performed during irradiation of the sample. Our previous findings [2] were confirmed. Typical values for the light-induced enhancement of the specific activity were in the range of 10 to 20%.

Coherent enzyme activity induced by light

The well accepted model of the enzyme activity is as follows: a substrate molecule binds to the enzyme and after the catalytic cycle, including the release of the product, the enzyme is ready to bind a new substrate molecule. The catalytic cycle of one enzyme complex is uncorrelated with the catalytic cycle of another enzyme complex (i) if there exists no interaction between the enzymes – there is no evidence for a self organization of the catalytic active P-450:NADPH-P-450 reductase complex – or (ii) if there is no external triggering signal. The uncorrelated catalytically working molecules will be referred to as the incoherent enzyme activity since there is no phase relation between the cycles of the working enzymes. For this situation, the product concentration increases linearly in time as shown in Fig. 2 ($t < 10$ s). Every enzyme in the test tube works independently of the working state of the other enzymes. Now let us consider that all the catalytic cycles have a fixed and constant phase relation. In this case the product concentration increases with time in a step function and not linearly as in the case of uncorrelated cycles. We achieved this result by triggering the cycles by periodic irradiation of the reaction mixture as shown in Fig. 2 ($t > 10$ s).

After the first flash, the fluorescence intensity still increases linearly in time but on an elevated level. After several flashes, the fluorescence intensity is pronounced and the recording shows very flat parts (as already mentioned the horizontal line as a part of the step function indicates that the cycles of all the enzymes are correlated). The predicted step function, if all the working enzymes are in phase, would be a step of 3.3 pmol 7-hydroxycoumarin (= 22% × 15 pmol P-450) followed by a horizontal line. If the first flash is followed by further flashes, as shown in Fig. 2, a large fraction of all the active enzymes in the test tube are working in phase. This result was obtained by evaluating two different parts of the step function: (i) the step hight and (ii) the finite slope after the step. The extrapolated concentration step hight, h, after a flash divided by the maximal step hight, h_{max} (= 3.3 pmol), yields the fraction "in-phase working enzymes". The finite slope of the increase in product concentration, dc/dt, divided by the slope obtained without step, h_{max}/T, yields the fraction of the "out-of-phase working enzymes". For example about 46% of the active enzyme complexes worked in phase determined after the fifth flash (43% determined from the step and 50% determined from the slope).

In consequence we performed a further experiment which will demonstrate directly the behaviour of free running "in-phase working enzymes" (Fig. 3). It is a similar experiment as already described in Fig. 2. First the product is formed without irradiation resulting in a linear increase of the product concentration (not shown in the figure, see Fig. 1). Then light flashes of 0.1 s were applied with a repetition time of 1.32 s to slave the working enzyme complexes. The irradiation was stopped after the 10th flash. If there are "in-phase working enzymes" then the product concentration should increase in time with step functions. This behaviour was actually observed. The repetition time between two steps is the turnover time of the free running reaction cycle. We measured a time of 1.53 s. This corresponds to a turnover number of

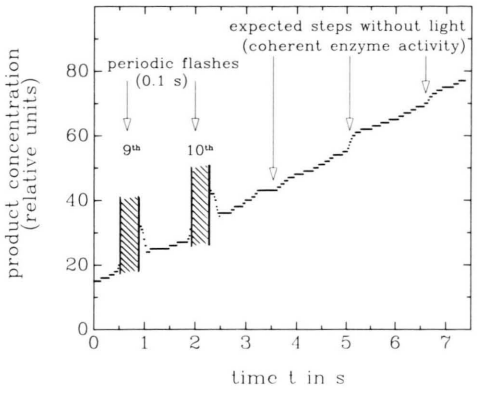

Fig. 3. Effect of stopping the periodic light pulses on the product formation of the 7-ethoxycoumarin-O-deethylase activity produced by a reconstituted P-450_{PB-B}: NADPH-P-450 reductase complex (conditions are given in the legend of Fig. 2). The last two of ten light pulses (duration of the pulse was 0.1 s, repetition time was 1.32 s) are shown. The arrows represent the rhythm of the applied light pulses (below 2 s) and the rhythm of the free running enzyme (above 2 s).

39 min^{-1} (optimum conditions). Müller-Enoch *et al.* [3] measured the maximum enzyme activity under optimum condition and found for the same enzyme and for the same substrate a value of 42 min^{-1} (obtained from the extrapolated enzyme activity for infinite high reductase concentration) and a value of 39 min^{-1} (obtained from the extrapolated enzyme activity for infinite high substrate concentration). The comparison of these two types of experiments shows that it is possible to measure the maximum catalytic activity of a given enzyme under conditions which are not optimum. It is worth-while to mention that the free coherently working enzyme provides direct evidence for the turnover number of the investigated enzyme. However, the classical methods for determination the turnover number need optimum conditions and it is very hard to perform experiments where all the essential concentrations can be extrapolated to infinity.

We obtained the following results: (i) a large fraction of 2×10^{12} working enzyme (= 0.24×15 pmol) complexes can be slaved by the light so that they work in phase. (ii) Only a small number of flashes are necessary to obtain a large fraction of "in-phase working enzymes". (iii) The cycle time can be measured and thus the turnover number under optimum conditions can be calculated.

Principles for slaving the enzymes in the cuvette

The cycle time or the characteristic time of the working enzyme is the time required for a single catalytic cycle and can be obtained by taking the inverse of the maximum enzyme activity under optimum conditions. We obtain for P-450_{PB-B} a turnover time, τ, of 1.54 s (39 min^{-1}). This value can be used as a starting position for the repetition time of the light pulses. If the repetition time, T, is greater or equal than the catalytic cycle time, τ, we expect, that most of the enzymes are working "out of phase". There is only a small step in concentration of the product after every flash and then the concentration increases linearly as in the non-irradiated case. After several flashes we did not find, that a large fraction of the enzymes are working still in phase. The other extreme case is where the repetition time is very short compared with the turnover time. We also expect no large fraction of "in-phase working enzymes". Obviously, there exists an optimum repetition time where the largest fraction of the enzymes can be brought to work in phase. If there is any chance to correlate the cycles in the test tube, then the repetition time has to be slightly smaller than the catalytic cycle time. This was our starting position and the found optimum repetition time was 1.32 s.

Lifetime of the product-enzyme complex

In the previous publication [2] we showed *via* the action spectrum that there are several possible stages at which light can accelerate the turnover time: (i) during transfer of the second electron to the complex, (ii) during formation of the ferric dioxygen complex with the release of a water molecule, and (iii) during the dissociation of the enzyme-product complex. Our flash experiments showed, that the product concentration increased stepwise after the light irradiation. From this step function we can conclude that the light has no accelerating effect during 1.0 s after the cycle has started. Our hypothesis is that the product dissociation is rate limiting and the light influences this process.

The time difference between cycle time, τ, and the optimum repetition time, T, for the light flashes is a lower limit for estimating the light-induced enhancement of the enzyme activity. Without light the cycle time is τ = 1.54 s. The cycle time of the

slaved enzymes is $T = 1.32$ s. The enhancement of the enzyme activity by shortening the cycle time is $(\tau - T)/T$. An enhancement of 14% is obtained ($T = 1.32$ s and $\tau = 1.54$ s) if every working enzyme has the possibility to absorb a light quantum. The light-induced enhancement determined from Fig. 2 is about 17%.

How can we understand the light-induced acceleration of the product release? Let us start with a simple reaction like $A \times B \rightarrow A + B$ with the rate constant k_{+1}. The metastable equilibrium distance between the two molecules A and B is described by a local minimum in the potential curve. The absolute minimum of the potential curve is reached when the two molecules are separated. There is a potential barrier between the local and the absolute minimum. The probability to pass over the barrier increases with increasing temperature, since the molecular vibration increases. This simple model can explain the temperature behaviour of the rate constant (Arrhenius plot). The energy of activation is reported to be 14 kJ · mol^{-1} [3]. We estimated the energy of activation from three temperatures to be between 10 and 18 kJ · mol^{-1}. The situation changes drastically if light is absorbed. The excited product $A \times B'$ has another potential curve. This can be explained in two ways: (i) the potential curve has no local minimum and is therefore repulsive for the two molecules, resulting in a very fast dissociation, (ii) the potential curve has a local minimum but the barrier to the dissociated state is small and the lifetime of the excited state is much shorter than that of the ground state.

Turnover number, catalytic cycle time, and enzyme activity

The specific enzyme activity for a given substrate is a function of (i) the concentration of the substrate itself, (ii) the concentration of P-450, (iii) the concentration of the P-450 reductase, and (iv) the concentration of NADPH. The optimum catalytic activity of an active P-450 : NADPH-P-450 reductase complex for a given substrate can be obtained under optimum conditions (all concentrations are very high, and extrapolation to infinite high concentration are done [3]). The maximum enzyme activity, V_{max}, of the reconstituted $P\text{-}450_{PB\text{-}B}$ and NADPH-P-450 reductase (under optimum conditions) is reported to be 42 nmol 7-hydroxycoumarin · min^{-1} · nmol P-450^{-1} (Table I of ref. [3], infinite concentration of reductase) and 39 nmol 7-hydroxycoumarin · min^{-1} · nmol P-450^{-1} (Table II of ref. [3], infinite concentration of substrate). This maximum specific enzyme activity is identical with the turnover number. The maximum specific enzyme activity is also given by the reciprocal of the catalytic cycle time [16]. The specific 7-ethoxycoumarin-O-deethylase activity of the reconstituted system used was 9.2 nmol 7-hydroxycoumarin · min^{-1} · nmol P-450^{-1}. The fraction of formed complexes of P-450 and of P-450 reductase was calculated to be 0.24 if the measured dissociation constant, K_D, of 600 nM was used (Fig. 3 of ref. [3]). In the cuvette 24% of the enzymes are working with their maximum activity. 76% of the enzymes did not form a complex and hence did not show enzymatic activity. If this picture of the working enzymes is correct then it should be possible to measure the turnover time of the catalytic cycle even under experimental conditions which are not optimum. Here we have to mention that we are the first which were able to demonstrate directly the turnover time of an actively working enzyme (after slaving the enzymes by light pulses). The turnover time is a specific value characterizing the catalytic cycle of an active P-450 : NADPH-P-450 reductase complex.

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