ELEMENTARY TURNOVER MEASUREMENTS ON THE MONO-OXYGENASE SYSTEM FROM LIVER MICROSOMES: MEASUREMENT OF INITIAL KINETICS

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1. Introduction

The enzymatic mono-oxygenase system from liver and other mammalian organs has been studied intensively from pharmacological and biochemical viewpoints [1]. The terminal enzyme of this system, cytochrome P_{450}, to which substrate and oxygen bind can be differentiated physically by at least two different types of absorption spectra, particularly depending on the bound substrate, for a high spin and a low spin form of the haemoprotein and for the ferric or the ferrous state of the central iron atom. Moreover the whole mono-oxygenase system activity depends in many respects on substrates under different conditions, e.g. pretreatment of the animal with various inducers of the mono-oxygenase system, preparation of microsomes from different organs or incubation of the microsomal suspension with cosubstrates or partial inhibitors.

So far it cannot be decided how the variety of specific activities corresponds to a multiplicity of the cytochrome P_{450}. In principle one has to consider two basic factors for an estimation of the activity: 1) the turnover number of the enzyme and 2) the fraction of the whole spectrally detectable cytochrome P_{450} which binds a substrate molecule.

No direct continuous measurements of the turnover number of cytochrome P_{450} are available. The fraction of enzyme that binds substrate has been determined, with cyclohexane as substrate, to be 30 to 50% of the total spectrally detectable cytochrome P_{450}. Regarding the kinetics of reduction of cytochrome P_{450} an indirect estimate of the turnover number was made, which turned out to be of the order of 1 sec^{-1} [2]. For direct measurements of the turnover number coumarin has been proposed as a substrate because of the more promising detection limit of the fluorescent product 7-hydroxycoumarin, and preliminary discontinuous measurements by means of rapid mixing kinetics and flash photolysis experiments indicate a turnover number similar to that with cyclohexane [3]. We have developed a method for direct, continuous measurements of the initial kinetics of the enzyme system which is necessary for the determination of the 'turnover number' of cytochrome P_{450}. To be sure of the participation of cytochrome P_{450} in the hydroxylation of coumarin a photochemical action spectrum was taken.

2. Experimental

The experimental method of preparing the microsomes [4] and the method by which the photochemical action spectrum was taken [5] have been described.

The method by which a single catalytic cycle of cytochrome P_{450} can be detected is based on the photoreversibility of cytochrome P_{450} inhibition by carbon monoxide. In the presence of carbon monoxide and oxygen in the enzymatic assay a certain percentage of reduced cytochrome P_{450} is inhibited by carbon monoxide. The inhibited fraction depends upon the partial pressure ratio of CO and O_2 in the preparation. The active fraction is determined by measuring the activity as a function of the CO to O_2 ratio.

When the sample is irradiated by high intensity light at the absorption wavelength, 450 nm, the carbon monoxide molecule dissociates from the complex and makes possible the association of an oxygen molecule.

This mechanism is the basis for a photochemical action spectrum [6] which proves the involvement of cytochrome P_{450} in the hydroxylation of coumarin to 7-hydroxycoumarin [7].

2.1. *Flash-photolysis apparatus*

A high intensity illumination set (Schoeffel Instruments Corp.) with a 2.5 kW xenon lamp and a monochromator 'GM 250' is used as light source. The light is regulated by an electromagnetically driven photoshutter (Compur electronic-m, size 1; Prontor Werk Alfred Gauthier GmbH) and illuminates the cuvette directly through a light pipe (about 50% transmission in the visible range; Jenaer Glaswerke Schott & Gen., Mainz). The shutter is controlled by an electronic pulse generator which allows the shutter-time to vary from 10 msec to 10 sec; the flashes can be repeated n times (n from 1 to 10^3) with a definite interval from about 10 msec to 90 sec. The samples are irradiated with blue light (450 nm, band width 16 nm) and a total intensity of about 35 mW. The fluorescence of the reaction product is measured and recorded with an 'MPF 3' fluorescence spectrophotometer (Perkin-Elmer).

3. Procedure for continuous measurements of initial kinetics

There are two consequences of a reversible inhibition by carbon monoxide which is controlled in time:

(1) An elementary catalytic process becomes detectable, in that a large number of enzyme molecules can be brought into a temporal correlation by reactivating the inhibited enzyme molecules by irradiation, i.e. the appearance of nearly all active enzyme molecules is synchronizable.

(2) The enzyme molecules reactivated by photodissociation can be allowed to do only a few cycles or even one cycle by adjusting the timing of irradiation.

This stop and go procedure by switching the irradiation on/off can be repeated frequently without losing the synchronization effect at the start position.

The catalytic cycle can be scanned by varying the irradiation time. During irradiation the formation of the carbon monoxide complex is stopped for a definite time in the course of which the enzyme molecules are allowed to work without inhibition. The number of product molecules formed represent an integration of two functions. One function is the time distribution function of the reactivated enzyme molecules during irradiation. The time distribution function is determined by the statistical nature of the elementary reaction. It describes the temporal distribution of the reactivated enzyme molecules at a certain stage of the catalytic cycle, i.e. the stage of product release. The other function describes the relaxation of the reactivated enzyme molecules into their resting state, i.e. the carbon monoxide complex, as soon as the irradiation is switched off.

With sufficiently short irradiation times – so that no enzyme molecule is allowed to do more than one reaction cycle – product formation is determined by the processes following photodissociation of the enzyme-carbon monoxide complex up to the stage of product release; so these processes can be separated from the whole cycle.

From the number of product molecules formed the turnover time of active enzyme molecules can be calculated if the distribution function is taken into account. Thus instead of a unique turnover time we have a distribution function according to which the turnover times of the individual enzyme molecules are distributed on account of the statistical nature of the elementary reactions. Fig.1 shows the methodical procedure.

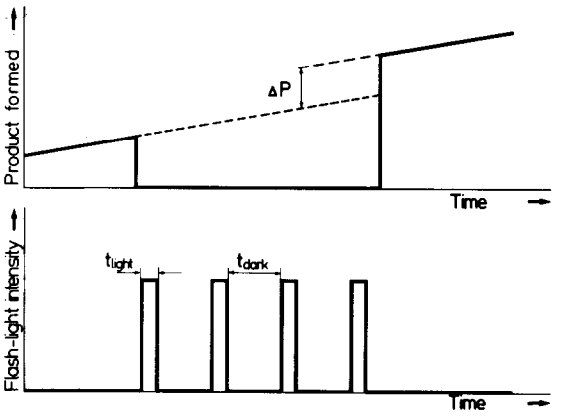

Fig.1. Schematic diagram of the measuring procedure. The diagram illustrates schematically the method of measuring described in the text. During the series of illumination cycles the product measurement was interrupted. The increase of the product can be determined in the way outlined. (t_{light}) Period of irradiation. (t_{dark}) Period of darkness. (ΔP) Product ($\simeq$ fluorescence) increase as a result of the flashes with the effect of the activity in darkness (zero product formation) being eliminated.

The product increase after a series of flashes in general may be written as

$$\Delta P_n = \Delta P\,(t_{light}, t_{dark}, n)$$

where t_{light} and t_{dark} are the period of irradiation and of darkness, respectively, and n is the number of flashes. If the period of darkness between two flashes is so long that the enzyme molecules return to their starting position the above relation simplifies to:

$$\Delta P_n = n \cdot \Delta P\,(t_{light})$$

So the product increase after n flashes equals the sum of n individual processes. To obtain a good signal to noise ratio it is advantageous to measure in this mode just above the lower limit of t_{dark}.

4. Results

As the inhibition factor of the coumarin hydroxylase depends on the concentration of carbon monoxide present in the microsomal suspension, one has to know this dependance for those experiments where photodissociation of the carbon monoxide complex takes place. Fig.2 shows the relative activity of coumarin-hydroxylase of rabbit liver microsomes as a function of the ratio of carbon monoxide to oxygen. Above a ratio of two, less than 10% of the activity without carbon monoxide will be found.

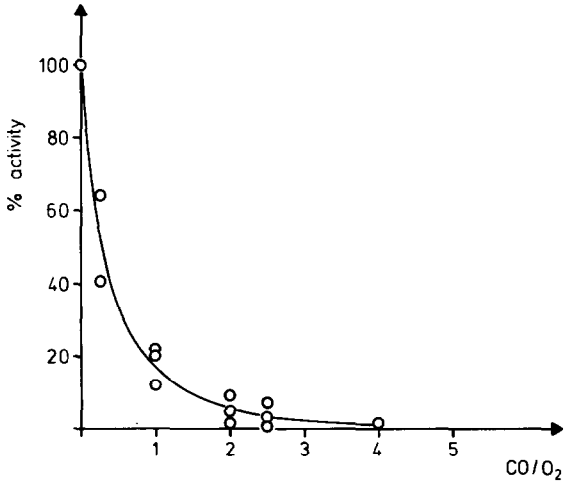

Fig.2. Relative activity of the coumarin hydroxylase of rabbit liver microsomes as a function of the CO/O_2 ratio. The enzymatic assay was as described below fig.3. The flasks were gassed continuously with a mixture of CO, N_2 and 10% O_2.

In rabbit liver microsomes coumarin is hydroxylated to the strongly fluorescent product 7-hydroxycoumarin (i.e. umbelliferon) among other products [8]. The photochemical action spectrum (fig.3) clearly shows the involvement of cytochrome P_{450} in the microsomal hydroxylation of coumarin.

A result shown in fig.3a which is of secondary importance with respect to the topic of this publication should be mentioned. In the control experiment where the microsomal preparation is irradiated in the absence of carbon monoxide a distinct peak around 435 nm is observable which might be interpreted either as a (partial) destruction of a shunt reaction, e.g. another active oxygen consuming process, or as a utilization of photon energy in an activation process which occurs somewhere in the electron transport chain leading to the hydroxylation of coumarin. This problem will be discussed elsewhere.

If we now apply the method to the investigation of an enzymatic cycle of cytochrome P_{450} on the hydroxylation of coumarin to umbelliferon we get the results as shown in fig.4. Within the measured interval from 0.05 to 3.5 sec of flash duration three intervals may be differentiated. In the first interval, up to about one second, the product formed increases with single flash duration. During the second interval, from about 1 to about 2.5 sec, it increases much less than in the first interval, whereas in the third range above 2.5 sec the product formed again increases with a higher rate.

We have presented a method to determine the initial kinetics of the mono-oxygenase system from liver microsomes. It has been applied to coumarin hydroxylase. We have pointed out the way in which the method may be used as a basis for a calculation of the elementary turnover time of the enzyme system.

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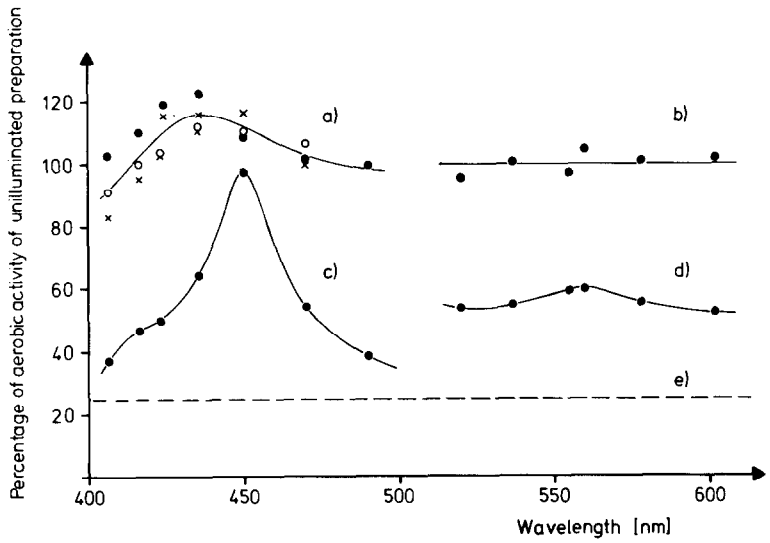

Fig.3. Photochemical action spectrum of the coumarin hydroxylase of rabbit liver microsomes. Each cuvette contained 3 ml 0.1 M Tris-HCl buffer pH 8.0, 5 X 10^{-3} M $MgCl_2$, 10^{-4} M coumarin, 2 X 10^{-4} M $NADP^+$, 2 X 10^{-3} M glucose 6-phosphate, (0.35 U glucose 6-phosphate) dehydrogenase. The incubation, 10 min at 25°C, was started by the addition of 0.1 ml rabbit liver microsomal suspension (30 mg protein/ml). (a) and (b) The cuvettes were gassed with air (•), 50% O_2 and 50% N_2 (○), 100% O_2 (×), (c) and (d) The cuvettes were gassed with a mixture of 20% O_2, 20% CO and 60% N_2. In experiments (b) and (d) the light intensity was about three times greater than in (a) and (c). (e) shows the activity of the unilluminated preparations taken in the experiments (c) and (d).

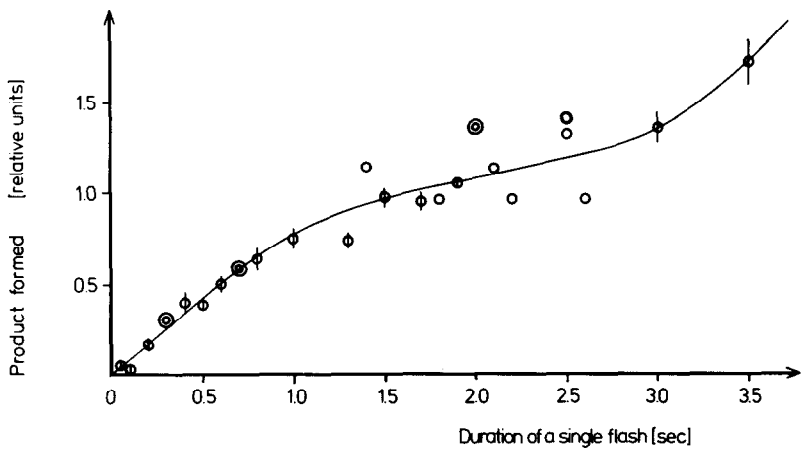

Fig.4. Product of the coumarin hydroxylase of rabbit liver microsomes formed during irradiation as a function of the irradiation time in the presence of CO. The standard assay of 1.1 ml total volume of 0.1 M Tris-HCl buffer pH 8.0 contains 75 μl microsomal suspension (7–10 mg protein/ml), 2.3 X 10^{-4} M NADPH, 4.6 X 10^{-3} M $MgCl_2$, 9.1 X 10^{-5} M coumarin, 10^{-4} M EDTA. The cuvette was gassed for 3 min with a mixture of 50% CO and 50% air (i.e. 10% O_2). After the addition of NADPH and of the microsomal suspension the cuvette was gassed again for 2 min and sealed afterwards. Because of the low quantity of product formed per cycle (about 10^{-13} mol in our present experimental conditions) irradiation was repeated 20 to 40 times. Between the single flashes the dark period t_{dark} was 5 sec. The specified 'product formed' is the product increase due to 20 flashes related to the product formed by an irradiation period of 60 sec. Measurements which were repeated three times and more are shown by their mean value and its standard deviation.

The experiments shown in figs 3 and 4 were performed at the Biochemisches Institut der Justus-Liebig-Universität, Gießen.

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