A FLASH-INDUCED REACTION OF A SYNTHETIC LIGHT-SENSITIVE SUBSTRATE WITH α-CHYMOTRYPSIN

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1. Introduction

High-resolution kinetic methods are very important for determining various intermediates in enzyme-catalyzed reactions and for measuring their interconversion rates [1, 2]. In the present communication an application of the flash method [3] for studying very rapid kinetics of enzymic reaction is developed. Unlike the established flow procedures [4] (cf. also [5]), the proposed method requires no quick mixing of the reagents. The substrate is formed in the presence of the enzyme by the action of light on a nonreactive substance, i.e. a light-sensitive pre-substrate.

The dead time of this procedure is determined by two parameters: (1) the flash time and (2) the time of photochemical conversion of the light-sensitive pre-substrate into the substrate. A conventional xenon flash technique allows one to have powerful flashes of 10^{-5}–10^{-4} sec in duration. The flash time can be further reduced by means of a laser device without decreasing the flash power (cf. [6]). The time of the photochemical reaction to form the substrate may also be sufficiently short.

Using this method we have studied the kinetics of the interaction between α-chymotrypsin (CT) and the *p*-nitrophenyl ester of *p*-nitro-*trans*-cinnamic acid (*trans*-NPNC). The light-sensitive pre-substrate of this reaction is the *p*-nitrophenyl ester of *p*-nitro-*cis*-cinnamic acid (*cis*-NPNC) which reacts with CT to form an acylenzyme at an exceedingly low rate compared to the *trans*-stereoisomer.

2. Experimental

Crystalline CT was obtained from the Leningrad meat packing plant [7]. *trans*-NPNC was prepared by the reaction of *p*-nitro-*trans*-cinnamoyl chloride with *p*-nitrophenol as decribed in [8]; m.p. 194°. To prepare *cis*-NPNC the solution of *trans*-NPNC in dimethylsulfoxide was UV-irradiated to obtain the photostationary mixture of stereoisomers [9]. Then *trans*-NPNC was hydrolyzed by CT and the *cis*-isomer (the substrate of low reactivity) was extracted from the aqueous solution by chloroform.

The flash photolysis installation used was as that described in [10]. The kinetics of *p*-nitrophenol formation in the flash-induced reaction were recorded by an oscilloscope. The oscilloscope sweep was triggered simultaneously with the pumping light. In a separate experiment it was shown that the time for *trans*-NPNC to reach the photostationary concentration is less than that of the flash.

The CT-catalyzed hydrolysis of *cis*-NPNC was followed spectrophotometrically at 400 nm using a Hitachi Perkin-Elmer 124 automatic recording instrument.

A kinetic description of enzymatic reaction set out in eqn. (1) has been given (cf. [11]).

$$E + S \overset{K_S}{\rightleftharpoons} ES \overset{k_2}{\underset{\searrow P_1}{\rightleftharpoons}} EA \overset{k_3}{\rightarrow} E + P_2 \qquad (1)$$

Equation (2) was used to obtain kinetic data on the enzymic hydrolysis reaction of *cis*-NPNC:

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$$\frac{d[P_1]}{dt} = \frac{k_2[E]_0}{K_s + [E]_0} \times ([S]_0 - [P_1]), \quad (2)$$

which is correct when $[E]_0 \gg [S]_0$. To determine the k_2 and K_s values, $[E]_0$ was varied from 2×10^{-4} M to 3×10^{-5}M at $[S]_0 < 10^{-5}$ M.

Enzymic flash-induced hydrolysis of *trans*-NPNC was studied under the following conditions: $[E]_0 = [S]_0 \ll K_s$, assuming [12] that $k_2 \gg k_3$. In this case the kinetics of formation of product P_1 (*p*-nitrophenol) may be described by equation (3). The value k_2/K_s was determined in terms of eqn. (3) at different initial concentrations $[E]_0 = [S]_0$ which were varied from 3×10^{-6} M to 1.5×10^{-5}M.

$$\frac{d[P_1]}{dt} = \frac{k_2}{K_s} \times ([S]_0 - [P_1])^2 \quad (3)$$

3. Results and discussion

The oscillogram (fig. 1) shows the kinetics of *p*-nitrophenol release in the flash-induced reaction of *trans*-NPNC with CT. Analysis of this kinetic curve in terms of eqn. (3) yielded tha k_2/K_s value presented in the table. The kinetic constants of slow enzymic hydrolysis of *cis*-NPNC are also presented in the table.

Table

	k_2 sec^{-1}	K_S mole/l	k_2/K_S l/mole sec
cis-NPNC[a,b]	1.7×10^{-2}	1.4×10^{-4}	1.2×10^2
trans-NPNC[a,c]	–	–	6×10^5

[a] pH 8.0 (buffer 0.1 M KH_2PO_4), 25°.
[b] 13.7 v.% dimethylsulfoxide.
[c] 19.2 v.% dimethylsulfoxide.

It can be seen from the table that the reactivity of the *trans*-isomer towards the CT active centre is more than three orders of magnitude higher than that of the *cis*-isomer.

The ratio of the rate constants of hydrolysis of *trans*-and *cis*-cinnamoyl-CT, $k_{3(trans)}/k_{3(cis)}$, also exceeds 10^3 [9]. The great difference in reactivity of the geometric isomers is likely to be due to the spatial configuration of the CT active centre. The data presented in the table confirm the concept of [13,14] that the β-substituted propionates are hydrolyzed in a conformation in which the β-aryl group and the hydrolyzing group are transoid.

At present we are studying CT-substrate interaction at resolution of 10^{-4} sec.

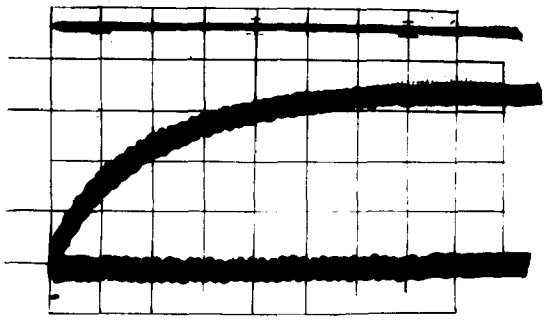

Fig. 1. The oscillogram of *p*-nitrophenol release in flash-induced reaction of *trans*-NPNC with CT. The experimental conditions are given in the table. The time scale is 50 msec per division. The horizontal traces show 0 and 100% of transmission (400 nm) respectively. The concentrations of the enzyme and the substrate (i.e. *trans*-NPNC to be formed after the flash) were 1.3×10^{-5} and 1.2×10^{-5} M respectively. The photostationary concentration of *trans*-NPNC was determined by the *p*-nitrophenol burst. The initial concentration of the pre-substrate (*cis*-NPNC) was 2.5×10^{-5} M.

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