Some evidence for the existence of two non-covalent complexes before the acylation step during the tryptic hydrolysis of Benzoyl-Arginine para-Nitroanilide.

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Received October 7, 1966

The occurence of more than one non-covalent enzyme-substrate complex in chymotrypsin catalyzed reactions had been supported by some previous results.Particularly the assumption of a second enzyme substrate complex characterized by conformational changes was proposed by Hess and al (1962,1965) for some reactions catalyzed by chymotrypsin. On the other hand, in our laboratory, some kinetic data obtained by Bechet (1965) and Chevallier and al (1966) working with trypsin in the alkaline pH range, seem to indicate that a group with a $pK_{app.}$ of 10 probably controls the acylation step but not the deacylation step and is not involved in the first Michaëlis-Menten complex formation; it was assumed that the occurence of a transconformation complex is dependent on this group.

The purpose of the present work was to detect by a rapid technique the transient steps occuring before the acylation step during the hydrolysis by trypsin of a specific amide substrate,Benzoyl Arginine para-Nitroanilide (BAPNA), the kinetics of this reaction being previously reported for steady state conditions by Erlanger and al. (1961)

For the experimental work, NOVO crystalline trypsin was used at a concentration of $3,6.10^{-4}$M and BAPNA (Mann Research Laboratories product) at a concentration of $1.27x10^{-4}$M in Tris buffer 0.05 M pH 8,4; with 1% of dimethylsulfoxide and $CaCl_2$ 0,02M. All the experiments were run at 21.5°C.

The kinetics of the reaction have been followed in non-steady state conditions by a stopped-flow apparatus. Its mixing device was built according to the details reported by Chance (1963). The stopped-flow apparatus was fitted to a Jobin and Yvon spectrophotometer, the photomultiplier output of which operated directly the amplifier

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of a Ribet-Desjardins model 242 A oscilloscope. The corresponding tracing and another reproducing the run of the pushing block, are both recorded by means of a Polaroïd camera. The mixing time was 5m sec.

As it has already been shown by Erlanger and al (I96I), the substrate 1-BAPNA (S), and the first reaction product, para-Nitroaniline (P_1) exhibit an isosbestic point at 34I mu. ($\varepsilon_s^{34I} = \varepsilon^{34I} = 7885$ cm^2/Mole).

From 3I0 mμ to higher wavelengths, there is no absorption either from the enzyme or from the second product, Benzoyl-Arginine (P_2). For $\lambda \geqslant$ 4I0 mμ, the changes in optical densities are only due to P_I release.

Kinetics have been followed at different wavelengths, from 3I0 mμ to 450 mμ. The kinetics at different wavelengths are reproduced on Fig. I. It is possible to distinguish three types of kinetics according to the value of λ, but a general pheno-

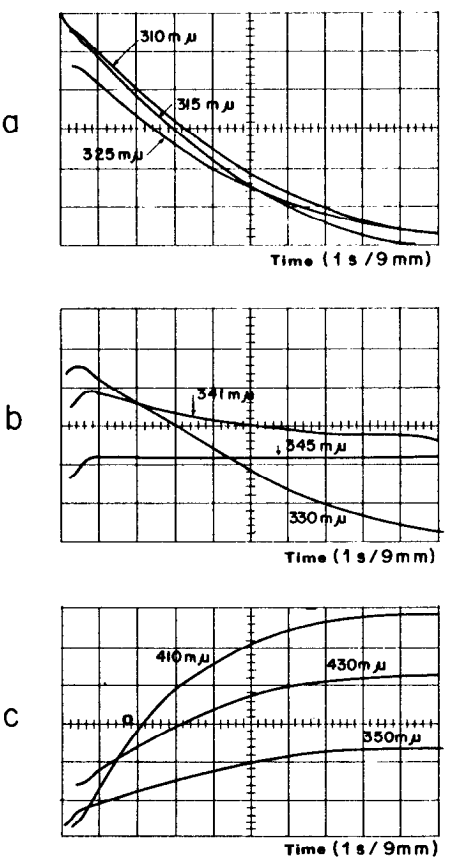

Fig. 1 : Oscilloscopic recording of transmittance changes at different wavelengths during the Trypsin catalyzed hydrolysis of Benzoyl d-l-Arginine-para-Nitroanilide (BAPNA) p : 8,4 ; T = 21,5°C

menon is observed : all kinetics present two different periods;the initial one is rapid (about 0,5 sec) and characterized by a small variation of optical density, and the second period is longer (about 10 sec.)The kinetics of the reaction obtained at the isosbestic point reported on Fig. 2 presents two characteristics : I) a maximum reached in about 0,5 sec. and 2) a decrease in absorbance to a final value smaller than the ini-

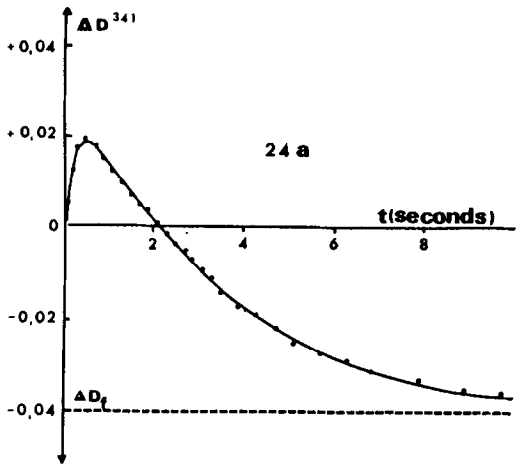

Fig. 2 : Changes of optical density at 341 mμ during the hydrolysis of BAPNA by Trypsin.

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tial value. From the shape of these kinetics one might assume two consecutive intermediates, before the acylation step, according to the scheme :

$$E + S \underset{k\text{-}I}{\overset{k_I}{\rightleftharpoons}} ES \xrightarrow{k_2} X \xrightarrow{k_3} ES' + P_I \xrightarrow{k_4} E + P_2$$

E,S,ES,X and ES', being respectively the enzyme, the substrate, the first Michaëlis-Menten complex, another intermediary complex, and the acyl-enzyme. P_I is the first product, para-Nitroaniline, and P_2 is the second one, Benzoyl-Arginine.

It has been possible to establish that in the final part of the reaction the disappearance of the X complex is a first order reaction characterized by a rate constant equal to that corresponding to the formation of the P_I product. The analysis of the kinetic data has been made for several values of the wavelength in the range 310 mμ-430 mμ and the value $k_3 = 0.34 \pm 0.08$ sec^{-I} was found in all cases.

The investigation made at the isosbestic point cannot be explained accor-

ding to the hypothesis of only one intermediary complex. It seems unreasonnable to believe that the increase of absorbance reaching a maximal value in a time of 0.5 sec. is due to the formation of the Michaëlis-Menten complex generaly assumed to be diffusion-rate controlled (Eigen and Hammes 1963). A rough calculation of the time required for the formation of the primary complex under our experimental conditions (enzyme and substrate concentrations) and assuming values of $10^6 M^{-1}$ sec^{-1} for k_1 and 10^3 sec^{-1} for k_{-1} is about 7×10^{-3} sec., which is the resolution time of the apparatus. The value of $10^6 M^{-1}$ sec^{-1} is certainly the lowest limit that it could be assumed for k_1 (Gutfreund 1955). The rate constants of formation of Michaëlis complexes seem at least higher by one or two orders of magnitude (Eigen and Hammes 1963). In such a situation, it is easy to understand that the initial value of the signal recorded (Fig. 2) is higher than the value obtained at the end of the spectrokinetic period investigated at the isosbestic point.

Some deviation from linearity can be observed when the absorbance changes to 410 mμ are ploted against the areas limited by the spectrokinetic at the isosbestic point (431 mμ) (Fig. 3). That means the spectrokinetic at 341 mμ results from two superimposed phenomena : first, the formation and the disappearance of the complexe X ; second the disappearance of an intermediate, the maximum concentration of which occurs at a time less

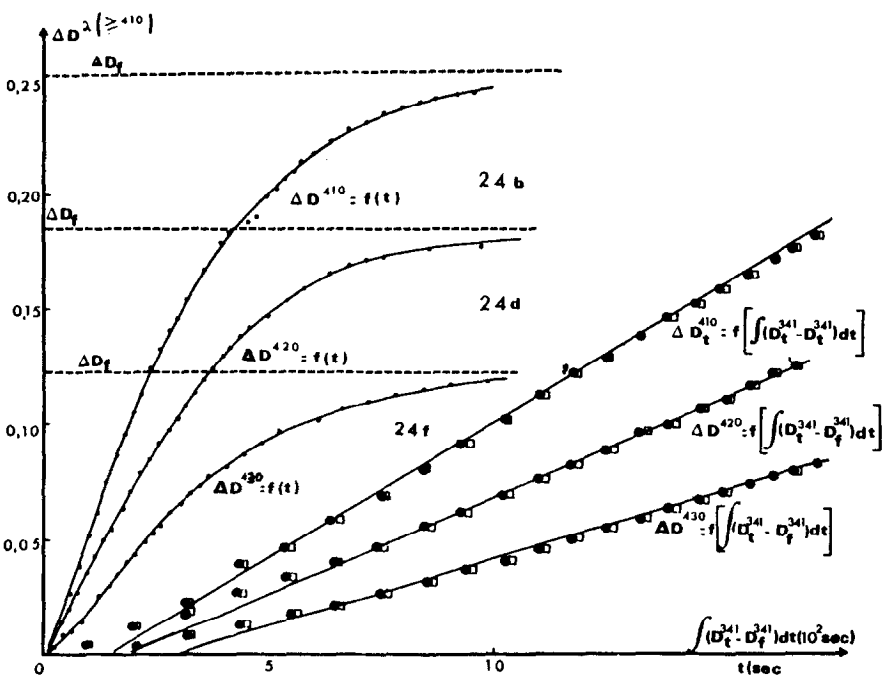

Fig. 3 : Changes of absorbance at 410 mμ , 420 mμ and 430 mμ 1:versus Time, corresponding to the release of para-Nitroaniline (——•——•——) 2/ versus the area limited by the spectrokinetic at 341 mu (○□).

than or equal to the mixing time.The first complex, the transformation of which gives the X complex, is probably the Michaëlis-Menten complex. The presence of two intermediates during the two first seconds of the reaction explains why the observed curves are not straight lines in the vicinity of the origin.

Erlanger and al. (5) have shown that the affinities of trypsin for the D and L-BAPNA isomers are nearly equal and under such conditions the trypsin-D-BAPNA association does not take part in the spectrokinetics observed at 341 mμ.

Further evidence for such an interpretation derives from the observation of some negative variation of optical density at 341 mμ although the rate of P_1 release is nearly constant in the interval time 0.6 to 1.8 sec. (Fig. 2 and 3).The concentration of the intermediate X has to be constant in this interval around its maximum concentration and the decrease of absorbance at 341 mμ cannot be attributed to X. It can be attributed to the disappearance of ES.

On the basis of this explanation it can be calculated that both ES and X are characterized by the same molar absorbancy at 341 mμ ($\varepsilon_{es}^{341} - \varepsilon_{X}^{341} = 10{,}000\ cm^2/Mole.$). Therefore it is possible to calculate the kinetics corresponding to ES and X separately, and the all over reaction at the isosbestic point as shown on Fig. 4.

The analysis of all the data obtained either at the isosbestic point or at different wavelengths values indicates that the reaction proceeds through the formation

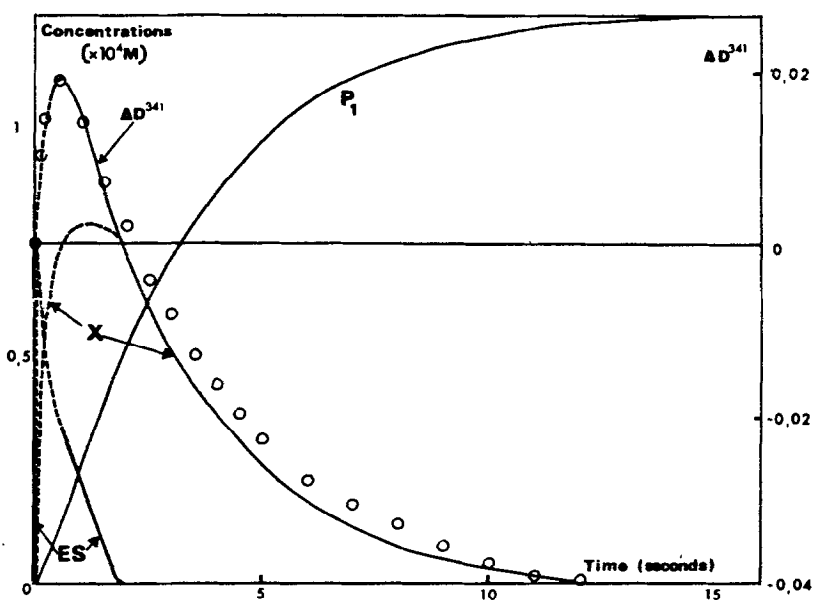

Fig. 4 : Kinetics of the appearance and desappearance of the intermediary complexes. The curve (Δ D^{341}) corresponds to the overall spectrokinetic at 341 mu. The circles (O) correspond to the experimental values.

of two consecutive complexes occuring before the acylation step. However it remains difficult to draw any conclusion about the nature of the X complex. The spectroscopic characteristics of this complex due to the chromophoric group of the substrate are not different from those of the first ES complex. The absorbancies of the substrate in the forms ES ans X are equal being higher than that corresponding to S alone. This is expected whith the localisation of the substrate in a more hydrophobic environnement resulting from its binding to the enzyme. In addition it seems to indicate that there is no strain in the substrate molecule during the transformation of ES to X, assumed for instance by the "rack theory". The assumption of X as a transconformation complex, complex involving some conformational changes in the enzyme molecule, seems however reasonnable. It is interesting to remark than the rate of formation of such a complex is about of the same order of magnitude as that corresponding to the allosteric transition (Kirschner, 1966).

This work was supported by a grant of the Cent National de la Recherche Scientifique. R C P n° 23.

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