Volume 268, number 1, 238–240 FEBS 08704 July 1990

Fixation of a highly reactive form of α-chymotrypsin by micellar matrix

A.V. Levashov[1], N.L. Klyachko[1], N.G. Bogdanova[1] and K. Martinek[2]

[1]*Department of Chemistry, M.V. Lomonosov Moscow State University Moscow, USSR* and [2]*Institute of Organic Chemistry and Biochemistry, Prague, Czechoslovakia*

Received 30 May 1990

Using reversed micelles of surfactants solvated by water-organic cosolvent mixtures as a matrix for enzyme entrapping, it is possible to fix the highly reactive α-chymotrypsin form. The reactivity of α-chymotrypsin towards nonspecific substrates increases to the extent comparable with that observed in reactions involving specific substrates.

α-Chymotrypsin; Conformation; Solvated reversed micelle; Substrate specificity

1. INTRODUCTION

A great number of different concepts and mechanisms of enzymatic catalysis have been suggested which account for both high reactivity and substrate specificity of enzymes in terms of enzyme-substrate interactions (see, for example, [1,2]). The thermodynamic background of all these concepts is the same: the gain in the free energy of the substrate binding by the enzyme is used for decreasing the activation barrier during subsequent chemical steps [1].

The kinetic role of the formation of the enzyme-substrate complex exemplified by α-chymotrypsin was investigated in detail by Berezin and Martinek [1,3,4]. The molecular mechanism of the catalysis involves the (self)assembly of the catalytic site of the enzyme as a result of conformational and solvation rearrangements in the substrate-enzyme complex [1,3,4]. The simplest kinetic scheme describing such a situation is shown in Fig. 1 where two equilibrium forms of the enzyme, the highly reactive one and the one of low reactivity, are designated by symbols T and R, respectively. In the absence of the substrate, the equilibrium is shifted towards form R while the specific substrate shifts it towards form T due to the multipoint interaction of the substrate molecule with the sorption site of the enzyme active center, which is more pronounced in the case of the form T. As a result, the groups constituting the catalytic site become rigidly fixed in a certain conformation. In other words, for the effective functioning of the catalytic assembly, the freezing of the conformational mobility or at least of the active center is necessary. In these terms, the catalytic activation of the enzyme can be regarded as a transition from the relaxed state R with low activity to the highly reactive tense form T (Fig. 1, pathway A).

Such a transition, in principle, can be forced to occur in the absence of substrate by using a properly chosen matrix in which the enzyme molecule would be fixed (immobilized). In our opinion, one of the most promising molecular matrices which can regulate the conformational mobility of the enzyme is provided by the systems of reversed micelles of surfactants in organic solvents (see, for example, reviews [5,6] dealing with enzymes and enzymatic catalysis in the systems of reversed micelles (Fig. 1, pathway B)). The key point here is that the size of the polar inner cavity of the reversed micelle, into which a water-soluble enzyme such as α-chymotrypsin is entrapped upon solubilization, can be easily varied by changing the degree of hydration of the surfactant (the ratio of molar concentrations of water and surfactant) [7,8]. In this way, as demonstrated by many examples [9], one can change the catalytic activity of entrapped enzymes in a very wide range. The maximal catalytic activity is observed under conditions when geometrical dimensions of the enzyme molecule and micellar inner cavity are matching. This effect is accounted for by the freezing of the conformational mobility of the enzyme active center as suggested by the ESR study of the spin-labeled α-chymotrypsin in the system of reversed micelles of Aerosol OT in octane [10]. The degree of the conformational freezing can be influenced still further by substituting water in the inner cavity of reversed micelles by water-miscible organic solvents (designated as cosolvents) such as glycerol, diols and dimethyl sulfoxide [11]. The potentialities of this approach to the regulation of enzymatic activity have been discussed elsewhere [11,13]. In the present work we consider the following question of principal importance: if the micellar matrix is able to convert the enzyme into the

Correspondence address: A.V. Levashov, Department of Chemistry, M.V. Lomonosov Moscow State University, Moscow, USSR

Published by Elsevier Science Publishers B.V. (Biomedical Division)

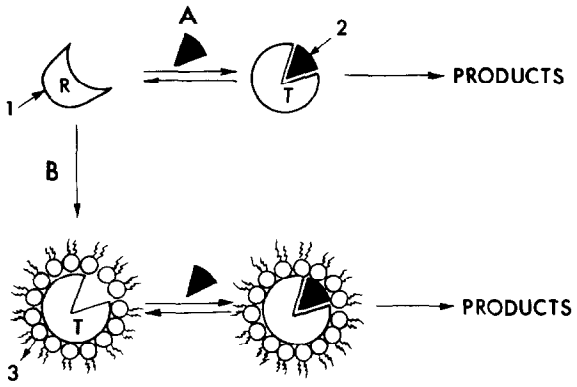

Fig. 1. Schematic representation of the conformational and solvation changes of α-chymotrypsin (1) induced by substrate (2) binding (pathway A) and/or by entrapment into a reversed micelle (pathway B).

highly reactive tense T form, is it possible to increase the reactivity of α-chymotrypsin towards nonspecific substrates to the extent comparable with that observed in reactions involving specific substrates?

To answer this question, we studied the hydrolysis of two series of α-chymotrypsin substrates, namely, *p*-nitrophenyl esters and *p*-nitroanilides of amino acids.

2. EXPERIMENTAL

2.1. *Materials*

α-Chymotrypsin (EC 3.4.21.1) from bovine pancreas was purchased from Reanal. Reversed micelles were prepared from diisooctyl sodium sulfosuccinate (Aerosol OT, AOT) purchased from Merck and used as received or from cetyltrimethylammonium bromide (CTAB, Chemapol) additionally purified as described elsewhere [11]. According to the IR data, the AOT and CTAB preparations contained 0.4 and 0.2 mols of water per mol of surfactant, respectively, and this was taken into account during calculations of the total water content in micellar systems. Water-free *n*-octane (Reakhim) was kindly provided by Dr A.A. Schegolev (Moscow University). Chloroform and glycerol (Reakhim) were purified according to the standard procedures [11]. 2,3-Butanediol (Merck) and dimethyl sulfoxide (DMSO) (Fluka) were used without further purification. The following substrates were used: *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTNA) (Sigma), *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA) (Serva), *N*-benzoyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (BOCTNPE) (BDH), acetic acid *p*-nitroanilide, trimethylacetic acid *p*-nitrophenyl ester (TMANPE) and caprilic acid *p*-nitrophenyl ester (CANPE) were prepared by Dr A.A. Schegolev (Moscow University).

2.2. *Preparation of stock solutions*

To 1 ml of 0.1 M solution of surfactant (AOT in octane or CTAB in chloroform/octane (1:1 v/v)) an organic cosolvent (0–50 μl glycerol, 2,3- or 1,4-butanediol, or DMSO) and aqueous buffered solution were added. The degrees of hydration $w = [H_2O]/[Surfactant]$ or solvation R = [Organic cosolvent]/[Surfactant] and the volume ratio water/organic cosolvent were determined from the amounts of organic cosolvent and buffered solution introduced into the system.

2.3. *The assay of the catalytic activity of α-chymotrypsin*

Hydrolyses of BTNA and SPNA were studied in the system AOT-water/2,3-butanedioloctane. The typical experiment was as follows. The solution of surfactant (1 ml) containing a water-organic mixture was mixed with 1–3 μl of 40–150 mM substrate solution in acetonitrile/dioxane (1:1 v/v). The reaction was initiated by addition of 1–5 μl of 1–4 mM enzyme solution in 50 mM Tris-glycine buffer (pH 8). An appropriate amount of the water-miscible organic cosolvent was also added to ensure the proper ratio water/organic cosolvent in the inner cavity of micelles. The reaction progress was followed spectrophotometrically (Beckman 25) at the wavelength of maximal *p*-nitroaniline absorption (380 nm). The value of ϵ_{380} was practically independent of the degree of hydration, the nature of surfactant, and concentrations of the components of the inner cavity, and was determined to be 9000–11000 $cm^{-1} \cdot M^{-1}$. The maximal rates of enzymatic reactions (the acylation was the rate-limiting step with the rate constant k_2) were determined from the Lineweaver-Burk plots. The hydrolysis of acetic acid *p*-nitroanilide was registered in the system AOT-water/2,3-butanedioloctane at the ratio H_2O/2,3-butanediol = 0.5/99.5 v/v, $w = 0.50$. The enzyme and substrate concentrations were 1 and 200 μM, respectively.

Hydrolyses of BOCTNPE, TMANPE, and CANPE were carried out in the system CTAB-water/DMSO-octane/chloroform. To 1 ml of 0.1 M CTAB solution containing a water-organic cosolvent mixture 2 μl of 0.1–0.5 M substrate in acetonitrile/dioxane (1:1 v/v) or DMSO and 1–2 μl of 3–5 mM α-chymotrypsin in 50 mM Tris-glycine buffer (pH 8) were added. The reaction was followed spectrophotometrically at 380 nm as described above. The rate constant k_3 of the rate-limiting deacylation step was evaluated under conditions of the saturation of the enzyme with the substrate.

The hydrolysis of BOCTNPE was run at pH 9.0, the concentrations of the substrate and enzyme being 10 μM and 3–6 nM, respectively.

The rotational frequencies (ν) of the spin label in the active center of α-chymotrypsin as measured using ESR in reversed micelles solvated by water-miscible organic cosolvents were taken from [11].

3. RESULTS AND DISCUSSION

We have previously shown [10] that the most effective freezing of the conformational mobility of the enzyme (minimal rotational frequency of the spin label in the active center) in the system of hydrated reversed micelles is observed when the size of the inner cavity of the micelle exactly corresponds to the size of the entrapped enzyme molecule. It is under these conditions where the enzyme displays the highest catalytic activity. In the systems of reversed micelles solvated by water-organic mixtures we also observed [11,13] maxima on dependencies of the enzymatic activity on the degree of hydration of reversed micelles. Such dependencies are always bell-shaped and for every concentration of organic cosolvent there is an optimal (i.e. corresponding to the highest k_{cat} and lowest ν) degree of hydration of the surfactant at which the principle of geometric correspondence of the enzyme and solvated micelle is realized.

We determined the values of k_{cat} and ν under these optimal conditions. The optimal k_{cat} and ν values determined for α-chymotrypsin-catalyzed hydrolysis of BOCTNPE in the system CTAB-water/DMSO-octane/chloroform at different concentrations of a water-miscible organic solvent (DMSO) are given in Fig. 2. Similar plots were obtained for other α-chymotrypsin-catalyzed reactions (data not shown). As seen from Fig. 2, the increase in the concentration of DMSO results in the increase in the optimal k_{cat} and the decrease in corresponding rotational frequency of the spin label in the active center.

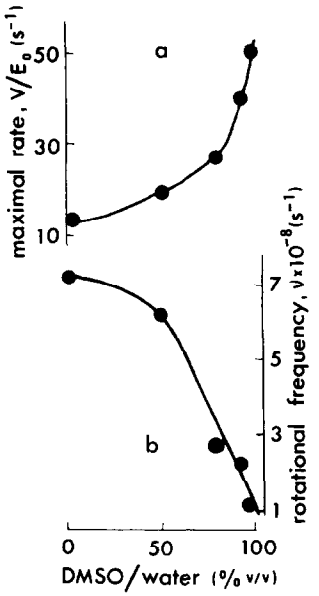

Fig. 2. The dependence of the maximal rate of hydrolysis of BOCTNPE catalyzed by α-chymotrypsin (a) and the rotational frequency of the spin label in its active center (b) on the concentration of DMSO (volume % towards water) in the system CTAB-H_2O/DMSO-octane/chloroform at water/surfactant molar ratios (w) optimal for catalysis: 4.30, 2.39, 1.18, 0.86, 0.48. Conditions: [CTAB] = 0.1 M, Tris-glycine buffer 0.05 M, pH 9.0, 22°C.

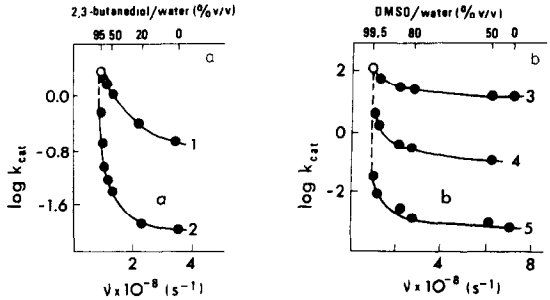

Fig. 3. The relation between the conformational mobility of the active center of α-chymotrypsin (rotational frequency of the spin label) and its catalytic activity in the hydrolysis of *p*-nitroanilides (a) and *p*-nitrophenyl esters (b): 1 = BTNA, 2 = SPNA, 3 = BOCTNPE, 4 = CANPE, 5 = TMANPE.

In order to compare the catalytic activity of α-chymotrypsin in hydrolyses of various substrates, in Fig. 3 we plotted the highest values of k_{cat} found at every concentration of organic cosolvent tested against the corresponding rotational frequency ν of the spin-label introduced into the active center of α-chymotrypsin. Inspection of Fig. 3 shows that both for *p*-nitroanilides (Fig. 3a) and *p*-nitrophenyl esters (Fig. 3b) the reactivity of α-chymotrypsin increases with decreasing conformational mobility. The range of the changes, however, is different when specific or nonspecific substrates are compared. For example, the reactivity of α-chymotrypsin towards BOCTNPE increases 5-fold on increasing DMSO up to 99.5%. At the same time, for the nonspecific substrate, TMANPE, the catalytic activity increases by 3 orders of magnitude. In other words, the difference in catalytic constant between 'good' and 'bad' substrates strongly diminishes, so that α-chymotrypsin becomes almost equally highly effective towards any substrate introduced into the system for which a drastic difference in specificity is observed in aqueous solution.

Based on these results we conclude that the interaction between entrapped enzyme molecule and surrounding micellar shell results in a rigid fixation of the tense conformation of α-chymotrypsin which thus acquires the ability to hydrolyze very effectively ester and amide bonds in different compounds including, probably, those that cannot be cleaved by α-chymotrypsin in aqueous solutions. As an example, we tested acetic acid *p*-nitroanilide which is extremely resistant to the enzymatic hydrolysis in aqueous solution. It was found that in the system AOT-water/2,3-butanediol (0.5/99.5 v/v)-octane α-chymotrypsin readily catalyzed the hydrolysis of this substrate. A possible reason for such behavior is that in the 'frozen' state the active site of the enzyme molecule entrapped into solvated reversed micelles is forced into the most favourable configuration, which otherwise can be created only as a result of the binding of a 'good' substrate (see Fig. 1).

In our opinion, the described method of stabilization of reactive forms of enzymes seems to be universal and can be used in enzymology both in fundamental studies (for the fixation of proteins in a certain conformation) and applied areas in particular in fine organic synthesis [14].

REFERENCES

[1] Berezin, I.V. and Martinek, K. (1977) Physical Chemistry of Enzymatic Catalysis, Vysshaya shkola (Russ.) 280 pp.
[2] Fersht, A. Structure and Mechanism of Enzymes Action.
[3] Berezin, I.V. and Martinek, K. (1970) FEBS Lett. 8, 261–262.
[4] Berezin, I.V. and Martinek, K. (1974) in: Structure and Function of Enzyme Active Sites (Russ.), Nauka, pp. 5–21.
[5] Martinek, K., Levashov, A.V., Khmelnitsky, Yu.L., Klyachko, N.L. and Berezin, I.V. (1986) Eur. J. Biochem. 155, 453–468.
[6] Luisi, P.L. and Magid, L.J. (1986) CRC Crit. Rev. Biochem. 20, 409–474.
[7] Zulauf, M. and Eicke, H.F. (1979) J. Phys. Chem. 83, 480–486.
[8] Eicke, H.F. and Pehak, J. (1976) Helv. Chim. Acta 59, 2883–2891.
[9] Martinek, K., Klyachko, N.L., Khmelnitsky, Yu.L. and Levashov, A.V. (1989) Biochim. Biophys. Acta 981, 161–172.
[10] Belonogova, O.V., Likhtenshtein, G.I., Levashov, A.V., Khmelnitsky, Yu.L., Klyachko, N.L. and Martinek, K. (1983) Biokhimiya (Russ.) 48, 379–386.
[11] Klyachko, N.L., Bogdanova, N.G., Martinek, K. and Levashov, A.V. (1989) Biokhimiya 54, 1224–1230.
[12] Zerner, B., Bond, R.P. and Bender, M.L. (1964) J. Am. Chem. Soc. 86, 3674–3679.
[13] Klyachko, N.L., Bogdanova, N.G., Martinek, K. and Levashov, A.V. (1990) Bioorg. Khim. (Russ.) 16, 581–589.
[14] Bogdanova, N.G., Klyachko, N.L., Kabakov, V.E., Martinek, K. and Levashov, A.V. (1989) Bioorg. Khim. (Russ.) 15, 634–637.