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On the Mechanism of Interaction of Organic Solvents with the Active Site of α-Chymotrypsin

E. A. Belyaeva, D. V. Gra, and N. L. Eremeev*

School of Chemistry, Lomonosov Moscow State University, Moscow, 119899 Russia;
fax: (095) 939-5417; E-mail: eremeev@enzyme.chem.msu.ru

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Abstract—Kinetic behavior of α-chymotrypsin in the reaction of hydrolysis of the N-acetyl-L-tyrosine derivatives was investigated in non-denaturing water–dimethylsulfoxide and water–ethanol mixtures. Similar specific interactions between the two solvents and the active site of α-chymotrypsin were shown to result in similar kinetic effects. It is proposed that the changes in the active site structure of the enzyme caused by the interaction with the organic solvents ("conformational isomer" formation) resulted in two parallel processes—acceleration of the acyl-enzyme formation step and a decrease in the deacylation rate. The possible molecular mechanism of this phenomenon and an adequate kinetic model describing the data are discussed.

Key words: α-chymotrypsin, dimethylsulfoxide, ethanol, mechanism of action

Experimental data on the behavior of proteinases (particularly α-chymotrypsin) in the presence of denaturing concentrations of organic solvents are rather contradictory. Different authors present different dependences of the catalytic constants on the concentration of organic solvents for the same systems. For example, for α-chymotrypsin in water–dimethylsulfoxide (DMSO) mixtures, the cases of the monotonic decrease [1-3], threshold dependences [4, 5], as well as activation effects [6] have been described.

Previously we demonstrated that the kinetic behavior of α-chymotrypsin in non-denaturing water–DMSO mixtures depended on the structure of the N-substituent of the amino group of the L-tyrosine-derived synthetic substrates [6]. Data on the hydrolysis of the N-benzoyl-substituted substrates were virtually identical, but the introduction of the acetyl group instead of the benzoyl group resulted in significant changes in the kinetics of the reaction while increasing DMSO concentration: a monotonic decrease in the catalytic constant was observed in the case of the ether substrate, while an activation maximum was characteristic for the nitroanilide substrate. Multipoint covalent immobilization of α-chymotrypsin into polyacrylamide gel (i.e., stabilization of the native conformation of the enzyme) removed the differences in the kinetic behavior of different substrates in the water–DMSO mixtures, exhibiting the dependences typical for the hydrolysis of the N-benzoyl-substituted derivatives of L-tyrosine by native α-chymotrypsin. Based on these data, the conclusion was made that the interaction of the enzyme with organic solvents resulted in conformational changes of the enzyme molecule.

Such a specific interaction apparently takes place in the sorption cavity of the N-acyl group of the substrate (S2 substrate-binding site of α-chymotrypsin according to the nomenclature of Schechter and Berger [7]). It is known that binding of the substrate in the S2 site of the active site of α-chymotrypsin is realized due to the hydrophobic interactions with Ile99 and/or Trp215 [8]. The Trp215 residue is situated close to the surface of the protein globule and accessible for the surrounding solvent [9]. The presence of the more hydrophobic N-benzoyl group in the substrate molecule provides more effective binding in the active site of the enzyme compared to the N-substituted analogs [10]. If the DMSO molecule is able to penetrate into the S2 sorption site, it can be assumed that the N-benzoyl group of the substrate is able to displace this molecule on the stage of the enzyme–substrate complex formation, while for the much less hydrophobic N-acetyl group such a displacement is unbeneficial in terms of energy. Thus, both the stabilization of the protein conformation and the higher sorption energy of the N-benzoyl group of the substrate defend the active site of the enzyme from the specific effect of DMSO. The cumulative data indicate that the N-benzoyl-substituted sub-

* To whom correspondence should be addressed.

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strates are hydrolyzed by native α-chymotrypsin, the active site of the enzyme retaining the native conformation, while the N-acetyl-substituted derivatives are hydrolyzed by its "conformational isomer".

The goal of the present study was to investigate kinetic properties of the hypothetical "conformational isomer" of the active site of α-chymotrypsin. A comparative study of the data on the hydrolysis of N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tyrosine *p*-nitroanilide in the non-denaturing water–DMSO and water–ethanol mixtures was performed (α-chymotrypsin denatures irreversibly in the presence of 4.2 M DMSO and 6.2 M ethanol [4, 5]). It should be noted that ethanol can take part in the reaction as an additional nucleophilic agent on the step of the acyl-enzyme deacylation, while DMSO is inert to the enzymatic hydrolysis.

MATERIALS AND METHODS

In the present work we used crystalline α-chymotrypsin (quality A) from Samson company (Russia), dimethylsulfoxide from Khimreaktivkomplekt company (Russia), and ethanol from Khant-Kholding company (Russia) without additional purification. N-Acetyl-L-tyrosine ethyl ester (ATEE) and N-acetyl-L-tyrosine *p*-nitroanilide (ATNA) were synthesized by the group of Organic Chemistry of the Department of Chemical Enzymology (School of Chemistry, Lomonosov Moscow State University). The purity of the synthetic substrates was no less than 99%. Other chemicals were of analytical grade (Reakhim, Russia).

The methods for titration of the active sites of the enzyme with *p*-nitrophenyl acetate are described in [11].

Assaying of the activity of α-chymotrypsin. The activity of native α-chymotrypsin was determined by measuring the initial rates of the hydrolysis of the substrates at pH 8.0 and 20°C. It was shown that the pH-optimum position did not change with the increase in the concentration of the organic solvents.

In the case of ATNA hydrolysis, the activity was monitored spectrophotometrically at 390 nm using an EPS-124 spectrophotometer (Hitachi, Japan). The reaction mixture contained 0.05 M sodium phosphate buffer; the substrate concentration was varied from 0.05 to 0.32 mM. The molar absorption coefficient of the product (*p*-nitroaniline) was determined in a separate experiment for each concentration of the organic solvent employed.

In the case of ATEE hydrolysis, the activity was monitored potentiometrically using an RT822 pH-stat (Radiometer, Denmark). The cell volume was 5 ml; the ionic strength was created by the addition of 0.2 M NaCl and 0.02 M $CaCl_2$. Concentration of the substrate varied from 2.5 to 10 mM.

The values of maximal velocities and Michaelis constants were determined from the dependences of the initial rates of the hydrolysis on the substrate concentration in the Lineweaver–Burk coordinates.

RESULTS AND DISCUSSION

Kinetics of the hydrolysis of different substrates by α-chymotrypsin is described by the three-step scheme [12, 13]:

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

where E is the enzyme, S is the substrate, ES is the enzyme–substrate complex, EA is the acyl-enzyme, and P_1 and P_2 are the basic (in our case ethanol or *p*-nitroaniline) and acidic (in our case N-acetyl-L-tyrosine) products of the enzymatic reaction, respectively. The catalytic constant of the reaction is defined as:

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3}. \qquad (2)$$

Depending on the leaving group, the rate-limiting step can be either the acyl-enzyme formation ($k_{cat} = k_2$, the nitroanilide substrate, ATNA), or its hydrolysis ($k_{cat} = k_3$, the ether substrate, ATEE).

Figure 1 demonstrates the dependences of the catalytic constants of the hydrolysis of the substrates by α-chymotrypsin on the concentration of the organic solvents in water–organic solvent mixtures. As seen from the figure, in spite of the significant difference between ethanol and DMSO in terms of the mechanism of the enzymatic reaction, the character of the dependences observed for the two solvents is similar—the monotonic decrease in the relative catalytic constant for the ether substrate and the activation effect for the nitroanilide substrate. Thus, we can conclude that for both ethanol and DMSO the same specific interactions with the active site of α-chymotrypsin take place, being revealed by similar kinetic effects.

Such a conclusion can be illustrated by the analysis of the data on ATEE hydrolysis (rate-limiting step is the deacylation). As mentioned above, ethanol as an additional nucleophilic agent can take part in the reaction at the step of the acyl-enzyme hydrolysis. Kinetics of such a reaction can be described by the following scheme [12, 13]:

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3[H_2O]} E + P_2 \qquad (3)$$
$$\downarrow \qquad\qquad \downarrow k_4[N]$$
$$P_1 \qquad\qquad E + P_3 .$$

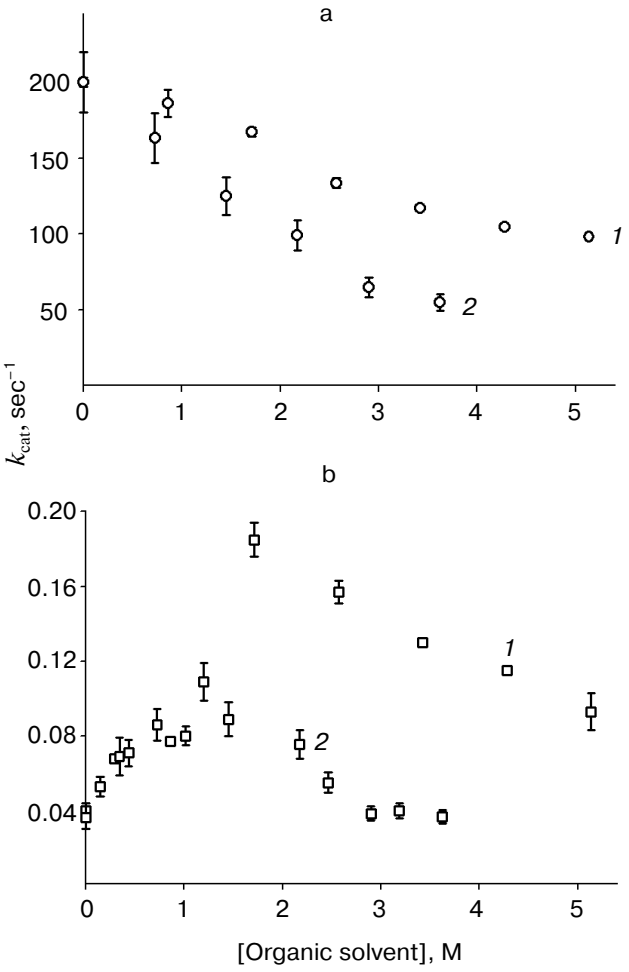

Fig. 1. Dependence of catalytic constants of the hydrolysis of ATEE (a) and ATNA (b) by α-chymotrypsin on the concentration of ethanol (*1*) and DMSO (*2*).

The use of the additional nucleophilic agent is a classic approach for separate determination of the constants of the individual steps of the hydrolysis of substrates by serine proteinases (for $k_2 > k_3$). Calculation of the experimental data on the ATEE hydrolysis in the water–ethanol mixtures according to this method (Fig. 1a, curve *1*) yields parameters that are in good agreement with the literature data (Table 1). However, the calculation with the use of the additional nucleophile is based on the decrease in the rate of the product P_2 formation, i.e., the chemical participation of the solvent in the reaction is not significant. Since the decrease in the catalytic constant of the ATEE hydrolysis by α-chymotrypsin is also observed in the water–DMSO mixtures (Fig. 1a, curve *2*), we decided to use this method formally for the water–DMSO mixtures. As seen from Table 1, the calculated parameters agree with the literature data reasonably well. Thus, the similarity in the kinetic behavior of the ATEE hydrolysis by α-chymotrypsin in ethanol and DMSO suggests a common mechanism of the effect of the organic solvents on the enzyme (not connected with its possible chemical participation on the step of the acyl-enzyme hydrolysis). The formation of the "conformational isomer" of the active site of α-chymotrypsin due to the specific interaction of the protein globule with the molecule of the organic solvent results in the decrease in the deacylation rate of the N-acetyl-L-tyrosine.

However, the activation effect observed for ATNA (rate-limiting step is acylation) in the presence of low concentrations of the organic solvents (Fig. 1b) can be accounted for the increase in the k_2 value only (see Scheme 1). But then, what is the reason for the subsequent decrease in the rate constant? As mentioned above, the decrease in the rate constant for ATEE hydrolysis corresponds to the decrease in the deacylation rate. Thus, it can be assumed that the changes in the structure of the enzyme active site in the presence of the organic solvent ("conformational isomer" formation) results in two parallel effects: deceleration of the deacylation step (decrease in the k_3 value) and acceleration of the acyl-enzyme formation step (increase in the k_2 value). This can result in the change of the rate-limiting step of the ATNA hydrolysis by α-chymotrypsin with the increase in the organic solvent concentration in the system, i.e., $k_3 \geq k_2$ and $k_{cat} \approx k_2$ in the range of low concentrations of the organic solvent (Fig. 1b, ascending branches), and $k_2 \geq k_3$

Table 1. Rate constants of the individual steps of the ATEE hydrolysis by α-chymotrypsin in water medium calculated from the data presented in Fig. 1a using the method of the additional nucleophile

Organic solvent	k_{cat}, sec^{-1}	k_2, sec^{-1}	k_3, sec^{-1}	k_4, sec^{-1}	K_S, mM
DMSO	205 ± 60	1460 ± 140	240 ± 90	1252 ± 532	3.7 ± 0.8
Ethanol	220 ± 10	3200 ± 1400	240 ± 30	840 ± 510	5.73 ± 0.03
Acetoxym*	130	3380	135	493	1.6 ± 0.4
1,4-Butanediol**	192	5000	200	—	17.2

* pH 7.8, 0.1 M NaCl, 25°C [12].
** pH 7.8, 0.1 M KCl, 25°C [13].

Table 2. Rate constants of the individual steps of ATNA hydrolysis by α-chymotrypsin in the maximum of its activity (the parameters were calculated from the data presented in Fig. 1b (descending branches of the dependences) using the method of the additional nucleophile)

Organic solvent	k_{cat}, sec^{-1}	k_2, sec^{-1}	k_3, sec^{-1}	k_4, sec^{-1}	K_S, mM
DMSO	0.10 ± 0.01	0.12 ± 0.01	0.61 ± 0.06	0.63 ± 0.07	0.736 ± 0.003
Ethanol	0.19 ± 0.08	0.24 ± 0.02	0.82 ± 0.07	0.33 ± 0.04	0.624 ± 0.005

and $k_{cat} \approx k_3$ in the range of high concentrations of the organic solvent (Fig. 1b, descending branches). In fact, the descending branches of the curves in Fig. 1b can be described adequately using the method of the additional nucleophile irrespective of the solvent nature (as mentioned above, this is possible only in the case $k_2 > k_3$), this allowing evaluation of the k_2 and k_3 constants in the maximum of the enzyme activity (Table 2).

To confirm the assumption concerning the possible change of the rate-limiting step in the case of the hydrolysis of the nitroanilide substrate, the mathematical modeling for the dependence of the catalytic constant of the ATNA hydrolysis by α-chymotrypsin on the concentration of the organic solvent was performed.

The dependence of the k_2 acylation constant for the ATNA hydrolysis on the concentration of the organic solvent was approximated by the linear dependence:

$$k_2 = \frac{k_{2\,max} - k_{2\,water}}{[N]^{max}}[N]^{max} + k_{2\,water}, \qquad (4)$$

where $k_{2\,water}$ is the value of the acylation constant in the absence of the organic solvent, $k_{2\,max}$ is the value of the acylation constant for the maximal k_{cat} value, $[N]^{max}$ is the concentration of the organic solvent corresponding to the maximal k_{cat} value for the ATNA hydrolysis (Fig. 1b). To plot the dependence, the calculated $k_{2\,max}$ value was used (Table 2), and the $k_{2\,water}$ value was taken equal to the $k_{cat\,water}$ value for the ATNA hydrolysis.

The dependence of the k_3 constant of the ATNA hydrolysis on the concentration of the organic solvent was approximated by the hyperbolic function:

$$k_3 = \frac{k_{3\,water}}{1 + \dfrac{k_{3\,water}}{[N]^{max} k_{3\,max}}[N]}. \qquad (5)$$

The $k_{3\,max}$ value was taken from Table 2, and the $k_{3\,water}$ value for the ATNA hydrolysis was taken equal to the k_3 value for the ATEE hydrolysis (Table 1), assuming that the rate of deacylation of N-acetyl-L-tyrosine-α-chymotrypsin in water is the same for both substrates.

The functions obtained for k_2 and k_3 were substituted into the equation for the catalytic constant (see Eq. (2)). Figure 2 shows the theoretical curves compared to the experimental values of the constants. As seen from the figure, the theoretical curves fit the experimental results

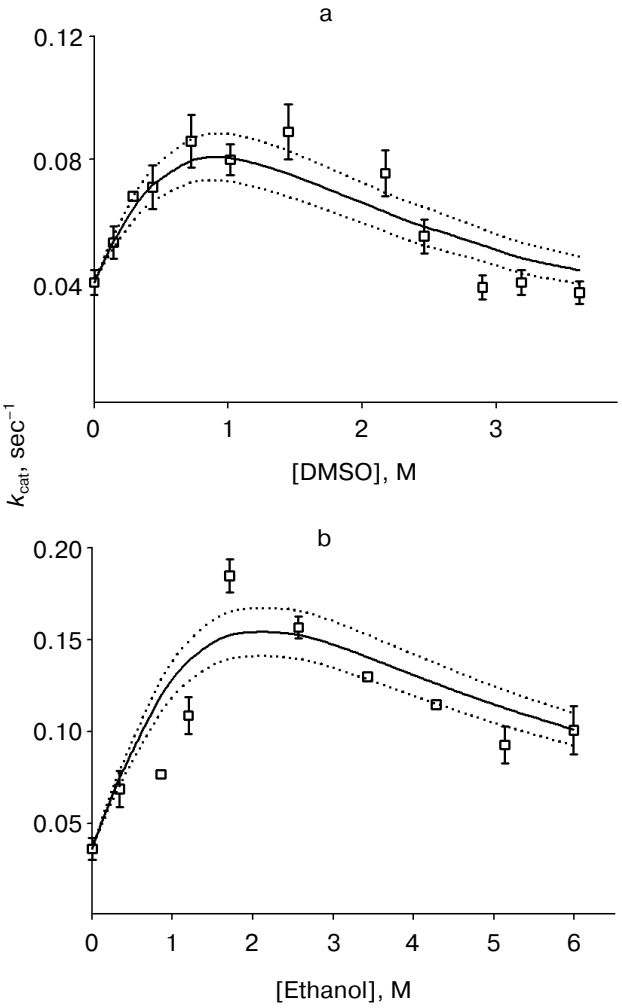

Fig. 2. Theoretical curves and experimental values of the kinetic constants for ATNA hydrolysis by α-chymotrypsin in water–DMSO (a) and water–ethanol (b) mixtures. The solid curve was calculated according to Eq. (2) with subsequent substitution of the k_2 and k_3 values from Eqs. (4) and (5), respectively. The limits of 10% confidence interval are shown by dotted lines.

reasonably well for both organic solvents. Thus, the interaction of organic solvents with the active site of α-chymotrypsin can really lead to the change in the rate-limiting step in the reaction of enzymatic hydrolysis of ATNA.

Comparative analysis of the experimental data and the results of mathematical modeling suggest that the specific interaction of molecules of the organic solvents with α-chymotrypsin result in formation of the conformational isomer of the active site of the enzyme. The characteristic feature of such a "conformer" is the parallel acceleration of the acylation step and deceleration of the deacylation step compared to the native conformation of the active site of the enzyme.

The molecular mechanism of the revealed phenomenon can be explained based on the concept of low-barrier hydrogen bonds [14-16]. According to this concept, formation of the low-barrier hydrogen bond between the Asp102 and His57 residues in the active site of α-chymotrypsin increases the basicity of His57 and improves its ability to abstract the proton from the Ser195 residue, this facilitating the nucleophilic attack of the carbonyl group of the substrate by the OH-group of Ser195 and formation of the tetrahedral intermediate compound [14, 15]. Moreover, the catalytic triad (Ser195, His57, Asp102) in this intermediate is stabilized by the low-barrier hydrogen bond between the amino acid residues, this decreasing the activation energy of its formation, i.e., the low-barrier hydrogen bond is necessary for acyl-enzyme formation [14-16]. An effect is known of "steric compression" arising in the active site of the enzyme on substrate binding promoting formation of the low-barrier hydrogen bond [15, 16], accelerating the step of the acyl-enzyme formation, and decreasing its deacylation rate. As it was shown while studying the interaction of peptidyltrifluoromethyl ketones with α-chymotrypsin, binding of a hydrophobic substituent in the S2-site of the enzyme active site stabilizes the low-barrier hydrogen bond between the His57 and Asp102 residues [16].

Thus, it can be assumed that binding of the more hydrophobic (compared to the N-acetyl group of the substrate) molecule of the organic solvent presumably results in the steric compression and, as a consequence, in stabilization of the low-barrier hydrogen bond between the His57 and Asp102 residues. The result is the increase in the acylation rate and the decrease in the rate of the acyl-enzyme hydrolysis that is revealed as the change in the rate-limiting step during ATNA hydrolysis by α-chymotrypsin.

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