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Review

Effect of tetrahydrofuran on the binding of the competitive inhibitor proflavin and the storage stability of bovine pancreatic α-chymotrypsin

The binding of the competitive inhibitor proflavin by bovine pancreatic α-chymotrypsin in water-tetrahydrofuran mixtures was studied in the entire range of thermodynamic water activities at 25°C. The data on the binding of proflavin were compared with the results on the storage stability of α-chymotrypsin in water-organic mixtures. An analysis of the concentration dependency of these characteristics demonstrated that, at low water activity values, the interprotein contacts in the enzyme formed during its drying largely govern its functional properties, while at high water activity, they are determined by the interaction of the enzyme with the organic solvent. The interplay of these two factors is responsible for the complex shape observed for the isotherm of binding of proflavin, with a maximum degree of binding being attained at medium water activity values.

Keywords: α-chymotrypsin / Enzyme activity / Storage stability / Organic solvents / Proflavin

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1 Introduction

This work is aimed at solving a topical problem–the elucidation of the physicochemical regularities of the functioning of enzymes in organic media with low water content. This problem is of considerable interest for non-aqueous enzymology, an innovation-promising scientific area [1–3]. The use of organic solvents as a reaction medium makes it possible to successfully conduct enzymatic reactions with hydrophobic compounds poorly soluble in water. Non-aqueous organic media provide the possibility of conducting industrially important synthetic reactions that do not occur in aqueous media (for example, peptide synthesis and esterification). The enzymatic catalysis in organic solvents is a competitive and cost-saving technology for producing substances of high optical purity.

On the other hand, reactions catalyzed by enzymes in organic liquids can take some hours. A high long-term stability of biocatalysts is necessary to make enzyme catalysis in organic solvents industrially successful. Some articles have been published on the operational stability of enzymes in low water containing organic liquids. Enzymes lost most of their activity exponentially upon their storage in organic solvents [4–6]. However, the mechanism of enzyme inactivation in low water containing organic solvents is still unclear. Therefore, it is clear that studying the regularities of biocatalysis in organic media makes it possible not only to optimize various biotechnological processes but also substantially extent the fundamental knowledge on the stability of enzyme macromolecules and on the forces maintaining the catalytically active conformation of enzymes under conditions of low water content.

At present, there is solid evidence that reactions catalyzed by α-chymotrypsin in water and organic media proceed via a single mechanism [7]. The classical competitive inhibitors of reactions catalyzed by α-chymotrypsin are aromatic compounds, including proflavin (3,6-diaminoacridine) [8–10].

One advantage of proflavin is its ability to form a 1:1 complex with the active site of the α-chymotrypsin molecule. Therefore, studying the regularities of the binding of a competitive inhibitor may be very informative for understanding the nature of the intermolecular forces that determine the state of the enzyme active site in the presence of organic media. For example, it was previously shown that a hydrophobic effect is responsible for the affinity of the competitive inhibitor to the enzyme in mixtures with high water content [8]. Binding of proflavin to chymotrypsin was studied in water-DMSO mixtures (with a water content of more than 40 vol % or a water activity of more than 0.65) [9]. Fink showed that the enzyme affinity to proflavin decreases with an increase

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in the content of the organic solvent. This effect was contributed to both the decrease of medium polarity and the competitive inhibition of the enzyme by DMSO. However, no attempt has been made to investigate the interaction of proflavin with chymotrypsin in organic solvents with low water content. These circumstances motivated us to study the effect of the organic solvent on the binding of the competitive inhibitor proflavin by α-chymotrypsin in the entire range of the thermodynamic water activity.

Tetrahydrofuran was chosen as the model solvent due to the following reasons:

(i) It is widely used in non-aqueous enzymology [11–13].
(ii) Tetrahydrofuran is a water-miscible organic solvent. Therefore, it is possible to study the effect of this low molecular organic substance on the enzyme activity over the whole range of thermodynamic water activity.
(iii) It is capable of forming strong hydrogen bonds with various hydrogen bond donors. However, in contrast to water, it has no evident hydrogen bond donating ability.

The aim of the present work was to study the effect of tetrahydrofuran on the binding of the competitive inhibitor and the storage stability of α-chymotrypsin in order to elucidate what intermolecular processes produce the main effect on the state and functioning of the enzyme at high and low water activities in organic media and to demonstrate how common are the regularities observed for polar acetonitrile and nonpolar dioxane [14, 15], the other hydrogen bond accepting solvents.

2 Experiments and methods

2.1 Materials

Bovine pancreatic α-chymotrypsin (Sigma, No. C 4129), essentially salt free; EC 3.4.21.1) and proflavin (P-2508, Sigma) were used without further purification. Tetrahydrofuran (reagent grade, purity > 99%) was purified and dried according to the recommendations [16]. Water used was double-distilled.

2.2 Enzyme storage stability

The enzyme stability was determined by measuring the enzyme activity after the storage in water-organic mixtures. The model process used was the hydrolysis of N-acetyl-*L*-tyrosine ethyl ester (ATEE) catalyzed by α-chymotrypsin. The measurements were performed on a Hiranuma Comtite-101 potentiometric titrator (Japan) in the pH-static mode at pH 8.0 and 25°C. The concentration of the substrate was 4.0×10^{-3} mol/L. During the course of the experiment, the pH value was maintained at a constant level by adding a titrant (a potassium hydroxide solution of known concentration), which neutralized the acid (N-acetyl-*L*-tyrosine) released during the hydrolysis. The kinetic curve obtained was the time dependence of the amount of the reagent spent for titrating the acid released. Each kinetic curve was reproduced not less than three times.

The reaction mixture was prepared as follows. A lyophilized α-chymotrypsin preparation with a humidity of 8.6±0.2% [g water/g enzyme] was immersed in an aqueous-organic mixture of a required composition and was incubated at 25°C for 1 h. This time period exceeded the time corresponding to the completion of the calorimetric heat effect accompanying the interaction of dehydrated proteins with pure organic solvents and water-organic mixtures [17]. The water content of the initial chymotrypsin samples was measured by drying using a microthermoanalyzer "Setaram" MGDTD-17S at 25°C K and 0.1 Pa until the constant sample weight was reached. The content of the α-chymotrypsin in the mixture was 1 mg/mL. Adding 100-μL aliquots of the aqueous-organic solution of α-chymotrypsin to the aqueous solution of the substrate, the enzymatic reaction was initiated.

A special experiment was performed to test the reliability of our method. For this purpose, the kinetic parameters of the enzymatic reaction in water at pH 8.0 and 25°C was calculated by using the Michaelis-Menten equation in the integral form: $K_M = 1.1 \times 10^{-3}(1.0 \times 10^{-4})$ mol/L, $V_{max}/[E]_o = 209$ (15) s^{-1}. Under similar conditions (25°C, pH 8.2), the authors of [18] obtained a $K_M = 1.2 \times 10^{-3}$ mol/L and a $V_{max}/[E]_o = 155\,s^{-1}$. These results show that our method is quite reliable.

The content of THF in the final reaction mixture did not exceed 0.5 vol. % (or 0.061 M) in all cases. Tetrahydrofuran can act as a competitive inhibitor like 1,4-dioxane [19]. This organic solvent has a structure similar to THF. This cyclic ether has four hydrophobic CH_2-groups like THF. A special experiment was performed to test this effect on the enzyme deactivation. For this purpose, the enzymatic reaction in the water-THF mixtures (0.5 vol % of organic solvent) was studied. No significant effect of THF on the kinetic parameters of the enzymatic reaction was observed. The K_M and $V_{max}/[E]_o$ values in the presence of 0.5 % of THF were 1.4 (0.2) mM and 201 (18) s^{-1}.

The inhibition effect of THF was quantitatively estimated. When the organic solvent acts as a competitive inhibitor, the Michaelis constant in the presence of the competitive inhibitor can be defined as follows:

$$K_{M,I} = \left(1 + \frac{[I]}{K_I}\right) K_M \quad (1)$$

where $[I]$ is the competitive inhibitor concentration [mol/L], K_I is the dissociation constant of the enzyme-competitive inhibitor complex [mol/L], $K_{M,I}$ is the Michaelis constant in the presence of the competitive inhibitor, and K_M is the Michaelis constant in the absence of any inhibitor [mol/L]. It can be concluded from Eq. 1 that the component $\frac{[I]}{K_I}$ is about 0.27 and the K_I value is 0.23. This is in agreement with the results of Bender et al. [19] who determined the dissociation constant of the chymotrypsin-competitive inhibitor complex for hydrogen bond accepting solvents. The K_I value for 1,4-dioxane was found to be 0.32 (pH 7.8, 25°C). For comparison, the K_I value for benzene (aromatic organic solvent, typical competitive inhibitor of α-chymotrypsin) is 0.0047 [8]. This means that the inhibition effect of tetrahydrofuran is weak in the studied mixtures.

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2.3 UV-visible spectrophotometry

These measurements were conducted on a Perkin-Elmer Lambda 35 double-beam scanning spectrophotometer at 25°C. In all experiments, the initial concentration of proflavin was 1.0×10^{-5} mol/L. The concentration of Tris-HCl buffer was 0.05 mol/L (the pH of the aqueous solution was 8.0). The initial state of α-chymotrypsin in the experiments with proflavin was a solid protein preparation with a humidity of 8.6 % [g water/g protein].

The degree of binding of proflavin by α-chymotrypsin (in the range of THF concentrations where the enzyme is soluble) was determined as follows. The interaction of proflavin with the active site of the enzyme results in a shift of the spectrum of the dye ($\lambda_{max} = 444$ nm in water) to longer wavelengths. The intensity of the difference spectrum of a proflavin solution with respect to the identical solution but containing α-chymotrypsin is a measure of the formation of the enzyme-proflavin complex. The amplitude of the maximum of the difference spectrum ($\lambda_{max} = 444$ nm in water) is proportional to the concentration of proflavin bound in the complex. In these experiments, the α-chymotrypsin concentration was varied from 6.2×10^{-6} to 1.9×10^{-4} mol/L.

At low and moderate water activities, at which α-chymotrypsin is insoluble, the pattern of the difference spectrum is different. In this case, the binding of proflavin by α-chymotrypsin manifests itself as a minimum in the difference spectra. The amplitude of the minimum is determined by the decrease in the concentration of proflavin in the solution because of its binding with α-chymotrypsin. The fraction of bound proflavin was calculated from the ratio of the amplitude of the minimum in the difference spectrum to the amplitude of the maximum in the spectrum of the initial solution. In these experiments, the α-chymotrypsin concentration was 1.9×10^{-4} mol/L.

2.4 Thermodynamic activity of water in organic solvent

Water activity [a_w] in organic solvent was calculated using the Eq. 2:

$$a_w = \gamma_w x_w \quad (2)$$

where x_w is the mole fraction of water in the solution and γ_w is the activity coefficient of water [in mole fraction]; the standard state is pure water).

Water activity coefficients [γ_w] were calculated using the literature data [20] on the vapor-liquid equilibrium according to the Eq. 3.

$$\gamma_w = \frac{y_w P_{tot}}{x_w P_w^o} \quad (3)$$

where y_w is the measured mole fraction of water in the vapor phase, P_t is the total pressure, P_o is the saturated vapor pressure of pure water at the same temperature and x_w is the mole fraction of water in the liquid phase.

3 Results and discussion

3.1 Enzyme storage stability

Fig. 1 shows the typical kinetic curves for the hydrolysis of ATEE catalyzed by α-chymotrypsin preliminary incubated in water-organic mixtures. The catalytic activity was characterized by the ratio of the extent of hydrolysis attained within 200 s with α-chymotrypsin incubated in a water-organic mixture to the same quantity measured using α-chymotrypsin incubated in pure water (see Fig. 1, Curve 1).

As can be seen from Fig. 2, THF affects the catalytic activity of the biocatalyst in a complicated way. At water activities from 0 to 0.4, the residual catalytic activity remains virtually constant, equal to ~60% compared to that observed after incubation in pure water. As the water activity increases from 0.4 to 0.9, the residual catalytic activity decreases sharply. At $a_w > 0.9$, the residual catalytic activity increases, approaching the level corresponding to pure water.

The K_m and V_{max} values for the hydrolysis of N-acetyl-*L*-tyrosine ethyl ester catalyzed by α-chymotrypsin preliminary incubated in water-tetrahydrofuran mixtures were calculated by using the Michaelis-Menten equation in the integral form. As can be seen from Figs. 2 and 3, the K_m values do not depend noticeably on the water activity in THF. On the other hand, the shape of the V_{max}-water activity curve is consistent with the storage stability dependence. The V_{max} values include two contributions: k_2 and E_T(the total amount of enzyme). Overall, this means that the changes in the V_{max} values occur probably due to the decrease in the amount of the catalytically active form of α-chymotrypsin during the incubation in water-organic mixtures.

3.2 Spectra of proflavin in water-THF mixtures

Figure 4A shows the spectra of proflavin in a water-THF mixture. As can be seen from Fig. 4A, the organic solvent

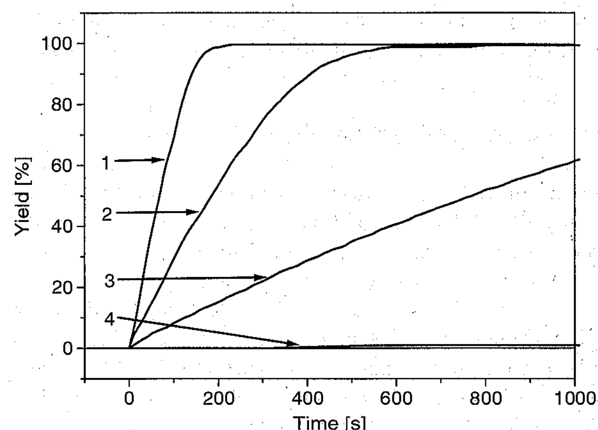

Figure 1. Typical kinetic curves for the hydrolysis of N-acetyl-*L*-tyrosine ethyl ester catalyzed by bovine pancreatic α-chymotrypsin preliminary incubated in water-tetrahydrofuran mixtures with various thermodynamic activities of water: (1) 1.0, (2) 0.01, (3) 0.7, (4) 0.87.

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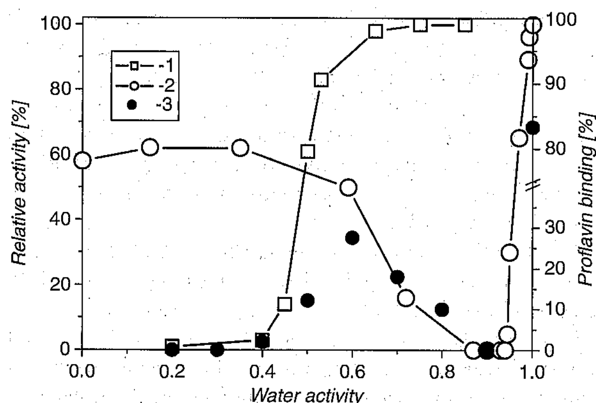

Figure 2. (1) Solid-state solvent-free hydrolysis of N-succinyl-L-phenylalanine-p-nitroanilide. Modified data from [22]. (2) Hydrolysis of N-acetyl-L-tyrosine ethyl ester by α-chymotrypsin preliminary incubated in water-tetrahydrofuran mixtures. Each experiment was performed 2–3 times. The experimental errors were 4–5%. (3) Binding of the competitive inhibitor proflavin by α-chymotrypsin in water-tetrahydrofuran mixtures. All values are the averages of three measurements. The experimental errors were 1–1.5%.

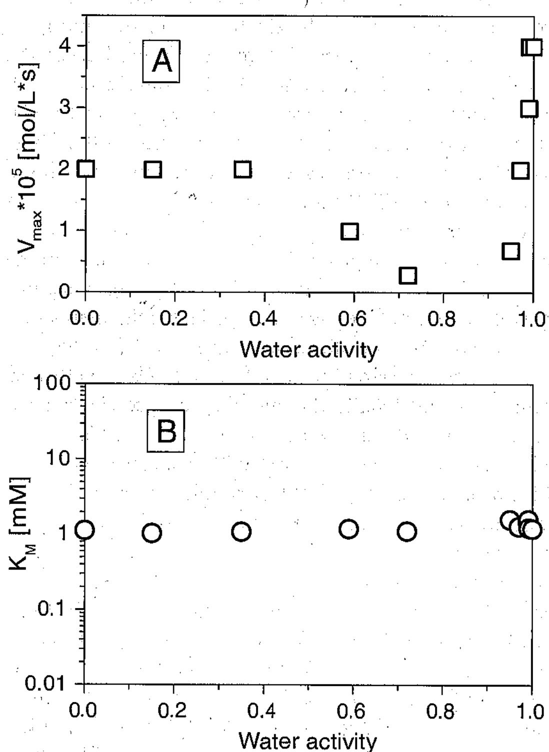

Figure 3. Dependences of the V_{max} and the K_m values on the water activity in tetrahydrofuran.

produces an appreciable effect on the shape of the proflavin spectrum. For example, at high water activities, the shape of the spectra and the position of the maximum are similar to those observed in pure water at pH 8.0. At $a_w < 0.5$, however, the shape of the proflavin spectrum is markedly different: a

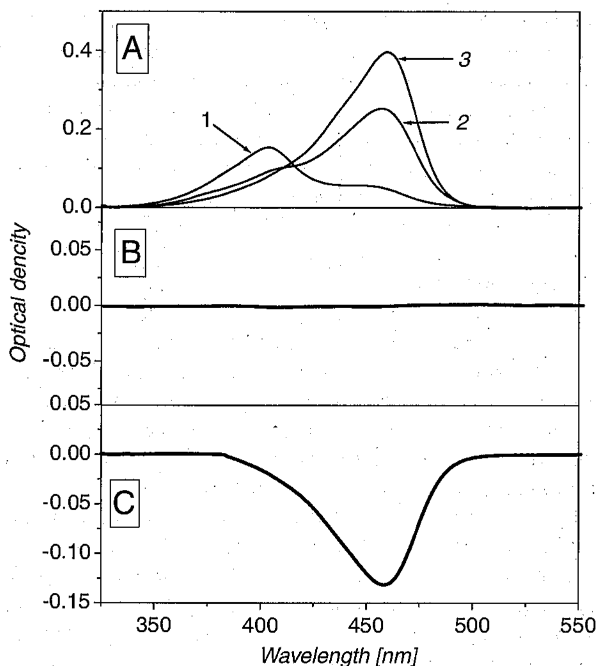

Figure 4. (A) Typical spectra of proflavin in water-tetrahydrofuran mixtures with various thermodynamic activities of water (1) 0.2, (2) 0.5, (3) 0.9 and (B, C) difference spectra of proflavin in the presence of α-chymotrypsin in water-tetrahydrofuran mixtures at a_w = (B) 0.2 and (C) 0.6. The initial concentrations of proflavin and enzyme were 1.0×10^{-5} and 1.9×10^{-4} mol/L, respectively. The Tris-HCl-buffer concentration was 0.05 mol/L.

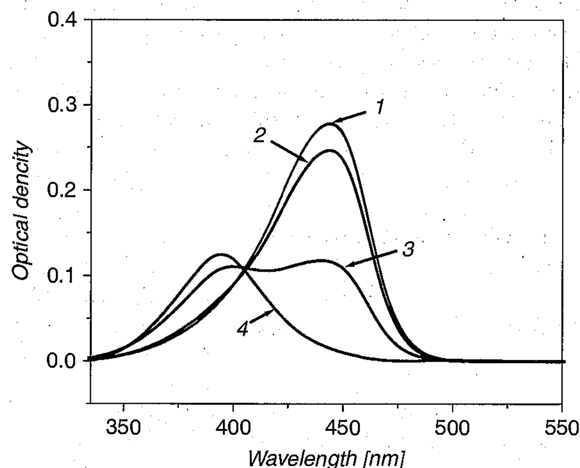

Figure 5. Typical absorption spectra of proflavin in water at various pH values: (1) 5.9, (2) 7.8, (3) 8.5, (4) 10.5.

new shortwave absorption band appears. The intensity of this band increases with the organic solvent concentration, while the intensity of the longwave band concurrently decreases. This behavior was interpreted as reflecting the coexistence of two forms of proflavin, protonated and deprotonated. As the water content in the water-THF mixtures decreases, the equilibrium shifts towards the deprotonated form. This conclusion is

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supported by the results of the following experiment. Fig. 5 displays the spectra of proflavin in water at various pH values. As can be seen, variations in the pH values are accompanied by the redistribution of the abundances of the protonated (predominant at low pH values) and deprotonated (predominant at high pH values) forms of proflavin, as it was in the case in water-organic mixtures.

3.3 Binding of proflavin in water-THF mixtures

In Fig. 4B, the difference spectrum of proflavin in the presence of α-chymotrypsin with respect to the initial solution of proflavin is shown in an aqueous-organic mixture with $a_w = 0.2$. As can be seen, the presence of the enzyme produces no effect on the proflavin spectrum. This means that in this mixture no binding of proflavin by the enzyme occurs. A similar spectrum was obtained for the mixture with $a_w = 0.3$.

Fig. 4C displays the difference spectrum of proflavin in the presence of α-chymotrypsin with respect to the initial solution of proflavin in an aqueous-organic mixture with $a_w = 0.6$. Similar spectra were obtained for mixtures with an $a_w = 0.4$, 0.5, 0.6, and 0.7. Within this range of water activities, α-chymotrypsin was insoluble. Therefore, the dip in the difference spectra was attributed to a decrease in the concentration of proflavin due to its binding by the enzyme.

The binding of proflavin in water-organic mixtures was qualitatively characterized by the absorbance at the isosbestic point (at 414 nm). This was motivated by the following circumstances. It is unknown in what form proflavin, protonated or deprotonated, is bound by the enzyme in aqueous-organic mixtures. On the other hand, at the isosbestic point, the absorption coefficients of both forms coincide. Correspondingly, by measuring the changes in the absorbance at 414 nm, one can determine the changes in the concentration of the competitive inhibitor in the solution irrespective of the bound form. The ratio of the absorbance at the 414-nm point of the difference spectrum to the absorbance at the isosbestic point of the spectrum of the initial solution is a measure of the fraction of bound proflavin at a given concentration of the enzyme. The degree of the binding of proflavin by α-chymotrypsin at a constant concentration of the latter (1.9×10^{-4} mol/L) as a function of the water activity is displayed in Fig. 2.

3.4 Effect of organic solvent on the storage stability and binding of the competitive inhibitor

The results regarding the storage stability of α-chymotrypsin and its ability to bind proflavin were interpreted within the framework of the model proposed in [21, 22]. According to this model, the dehydration of proteins results in the formation of strong intermolecular contacts via the establishment of hydrogen bonds and/or ionic bridges between the side polar groups of the protein (carboxyl, alcohol, and amino groups). As a result, the rigidity of the protein structure increases while an appreciable fraction of the protein polar groups of the dry protein, those involved in the formation of interprotein contacts, becomes incapable of acting as sorption sites. These changes manifest themselves through sorption hysteresis [21, 22]. Correspondingly, if these groups enter into the composition of the active site of the enzyme, they become incapable of interacting with the molecules of the substrate or the competitive inhibitor. As a result, α-chymotrypsin shows no catalytic activity in reactions of the solid phase hydrolysis at low water activities ($a_w < 0.4$) in the absence of an organic solvent (see Fig. 2) [23].

On the other hand, it was demonstrated that the stability and the structure of the dried proteins are substantially dependent on the ability of the organic solvent to form hydrogen bonds [24, 25]. Considerable structural changes and exothermic effects were observed only in solvents capable of forming strong hydrogen bonds. Consequently, knowledge of the tradeoff between the hydrogen bond accepting and hydrogen bond donating properties of the solvent is important for predicting the possible influence of organic molecules on the functional characteristics of the protein. Indeed, when the interprotein contact formed by hydrogen bonds is broken, a solvent (water or THF) molecule has to choose with which fragment it has to interact.

Water molecules can solvate both hydrogen bond accepting and hydrogen bond donating groups of α-chymotrypsin. By contrast, hydrogen bond accepting THF molecules will predominantly solvate the hydrogen bond donating group of the broken contact (the remaining hydrogen bond accepting group will be more effectively solvated by water molecules). Thus, THF molecules are incapable of breaking interprotein contacts in the absence of water. Correspondingly, the interaction of dry chymotrypsin with anhydrous hydrogen bond accepting solvent (like THF) is not accompanied by significant heat effects or structural changes [23, 24]. This means that, at low water activities, THF produces no appreciable effect on the state of the initially dehydrated enzyme. Therefore, a significant level of catalytic activity was observed after the incubation of CT in the aqueous-organic solvent at $a_w < 0.4$ (see Fig. 2).

Only above the threshold protein humidity ($h \sim 0.1$ g water/g enzyme or $a_w \sim 0.4$–0.5), the mobility of protein macromolecules increases markedly, the catalytic activity rises sharply, and the state of the secondary structure approaches that of the native protein [20, 11, 25–26]. It should be noted that the significant increase in the degree of binding of the competitive inhibitor and the catalytic activity of the enzyme in the reaction of the solid-phase hydrolysis were also observed at $a_w = 0.4-0.5$ (see Fig. 2). According to the proposed model, at low water activities, water molecules penetrate into the structure of the dried enzyme, break interprotein contacts, and hydrate the polar groups of these contacts. At low water activities, interprotein contacts play a negative role, hindering the formation of the active form of the enzyme. Thus, the stage of breaking interprotein contacts plays a key role in the behavior of proteins in organic media.

At $a_w > 0.5$, the catalytic activity of the enzyme and its ability to bind proflavin vary in a similar way, both passing through a minimum at $a_w \sim 0.8$–0.9. On the other hand, the enzymatic activity in the absence of the organic solvent shows no minimum at high water activities. This suggests that, at a high degree of hydration of the enzyme, when most of the interprotein contacts are already broken, the interaction with

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an organic solvent determines the functional characteristics of the enzyme. It was demonstrated [21, 22] that, in this range of water activities, the enzyme is denaturated by a hydrogen bond accepting solvent with the formation of intermolecular β-structures.

At moderate water activities, the degree of binding of proflavin (as demonstrated in Fig. 2) passes through its maximum, reflecting the interplay between the following processes. On the one hand, the hydration of the enzyme is already high enough, so that its conformation is close to the native one. On the other hand, some interprotein contacts remain unbroken, a factor that plays a positive role by hindering the denaturation of the enzyme by the organic solvent.

It is also remarkable that, as follows from Fig. 2 and the data reported in [14, 15], the shapes of the isotherms of binding of proflavin by α-chymotrypsin and the positions of the maximum degree of binding in THF, dioxane, and acetonitrile are similar. These nonelectrolytes possess identical hydrogen bond accepting abilities with respect to water, as indicated by the similarity between the enthalpies of specific interaction $\Delta H_{int}^{H_2O/S}(spec.)$ (−18.0 kJ/mole for acetonitrile, −19.3 kJ/mole for dioxane, −19.7 kJ/mole for THF [27]). For comparison, the enthalpies of specific interaction of water with benzene and DMSO equal −1.5 and −33.1 kJ/mole, respectively. This means that the formation of the enzyme-competitive inhibitor complex in moderate-strength hydrogen bond accepting solvents, such as THF, dioxane, and acetonitrile, exhibits similar regularities at low water activities.

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