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BIOCHEMICAL CHEMISTRY

Effect of Dioxane on the Binding of Competitive Inhibitor Proflavin and Catalytic Activity of Bovine Pancreatic α-Chymotrypsin

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Abstract—The binding of competitive inhibitor proflavin by α-chymotrypsin in water–dioxane mixtures over the entire range of thermodynamic activities of water a_w was studied. The data on the degree of binding of proflavin were compared to the results on the catalytic activity of the enzyme preliminary incubated in water–dioxane mixtures. An analysis of the behavior of the concentration dependences of these characteristics demonstrated that, at low a_w values, the behavior of the interprotein contacts in the enzyme formed during its drying largely governs its functional properties, while at high a_w values, they are determined by the interaction of the enzyme with the organic solvent. Interplay of these two factors is responsible for the observed complex shape of the isotherm of binding of proflavin, with the maximum degree of binding being attained at moderate a_w values.

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This work is a continuation of our systematic studies [1–3] aimed at solving a topical problem—elucidating the physicochemical regularities of the functioning of proteins in aqueous–organic media with low water content. This problem is of considerable interest for nonaqueous enzymology, an innovation-promising scientific direction [4–6]. The use of organic solvents as a reaction medium makes it possible to successfully conduct enzymatic reactions with hydrophobic compounds poorly soluble in water. Nonaqueous organic media provide the possibility of conducting industrially important reactions of synthesis that do not occur in aqueous media, for example, the synthesis of peptides and esterification. The enzymatic catalysis in organic solvents is competitive and cost-saving technology for producing substances with a high optical purity [4–6].

On the other hand, 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 fundamental knowledge on the stability of protein macromolecules and on the intramolecular forces maintaining the catalytically active conformation of enzymes under conditions of low water content.

At present, there is solid evidence that reactions catalyzed by the α-chymotrypsin enzyme in water and anhydrous 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 the competitive inhibitor may prove useful for understanding the nature of the intermolecular forces that determined the state of the active site of the enzyme in low-water organic media.

These circumstances motivated us to continue studies on the binding of competitive inhibitor proflavin by α-chymotrypsin. Dioxane, one of the solvents widely used in nonaqueous enzymology, was chosen as the model organic solvent [4–6]. Data on the binding of proflavin were compared to the results on the effect of dioxane on the catalytic activity of the enzyme. The aim of the present work was to study the concentration dependences of the efficiency of binding of the competitive inhibitor and the enzymatic activity chymotrypsin in order to elucidate what intermolecular processes produce the main effect on the state and functioning of the enzyme at high and low activities of water in organic media and to demonstrate how common are the regularities observed for acetonitrile [2], another proton-acceptor solvent.

EXPERIMENTAL

Materials. Bovine pancreatic α-chymotrypsin (EC 3.4.21.1, Sigma, C-4129, Type II) and proflavin (P-2508, Sigma) were used without additional purification. The catalytic activity of α-chymotrypsin in the preparation was 52 units per g of substance. The organic solvent was purified and dried as described in [11].

Determination of the enzymatic activity. The model process was the hydrolysis of N-acetyl-L-tyrosine ethyl ester (ATEE) catalyzed by α-chymotrypsin. The mea-

surements were performed on a Hiranuma Comitite-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. The constant level of pH was maintained by adding a titrant (a known concentration solution of potassium hydroxide), which neutralized the N-acetyl-L-tyrosine acid formed by the hydrolysis reaction. The kinetic curve was plotted as the time dependence of the amount of the reactant spent for titration of the acid released. Kinetic curves for each given set of conditions were measured twice at least.

The reaction mixture was prepared as follows. A lyophilized preparation of the enzyme with an initial humidity of 8.6 wt % (g water/g protein) was placed into an aqueous–organic mixture of required composition and incubated at 25°C for 1 h. The content of the enzyme in the mixture was 1 mg/ml. Next, adding a 100-ml aqueous–organic aliquot containing the enzyme, we initiated the enzymatic reaction. In all cases, the content of dioxane in the final reaction mixture was not less than 0.5 vol % (with a water activity of $a_w > 0.99$).

In special experiments, we checked the reliability of the method. For this purpose, using the linearized Michaelis–Menten integral equation, we calculated the kinetic parameters of the enzymatic reaction in water at pH 8.0 and 25°C: $K_m = 1.1 \times 10^{-3}$ M^{-1} and $V_{max}/[E]_0 = 209$ s^{-1}. Under similar conditions (pH 8.2, 25°C), the authors of [12] obtained $K_m = 1.2 \times 10^{-3}$ M^{-1} and $V_{max}/[E]_0 = 155$ s^{-1}, a result indicative of the reliability of the method we used.

UV spectrophotometric measurements. The degree of binding of proflavin by chymotrypsin in water–organic solvent mixtures was developed by us in [2]. The measurements were conducted on a Perkin-Elmer Lambda 35 double-beam scanning spectrometer at 25°C. In all the experiments, the initial concentration of proflavin was 1.0×10^{-5} mol/l, the concentration of Tris-HCl-buffer was 0.05 mol/l, and the pH of the aqueous solution was 8.0. The enzyme used in the experiments with proflavin was a solid preparation with a humidity of 8.6 wt % (g water/g dry enzyme), which was measured on a Setaram MGDTD-17S microthermoanalyzer by drying the sample at 25°C and 0.1 Pa to constant mass.

This method is based on the following principle. The interaction of proflavin with the active site of the enzyme results in shift of the spectrum of the dye ($\lambda_{max} = 444$ nm in water) towards the longwave region. Recording the difference spectrum of a proflavin solution with respect to the same solution but containing the enzyme makes it possible to determine the extent of formation of the enzyme–proflavin complex. The optical density of the difference spectrum at the maximum ($\lambda_{max} = 465$ nm in water) is proportional the concentration of proflavin bound in the complex. The concentration of the enzyme in these experiments was varied from 6.2×10^{-6} to 1.9×10^{-4} mol/l.

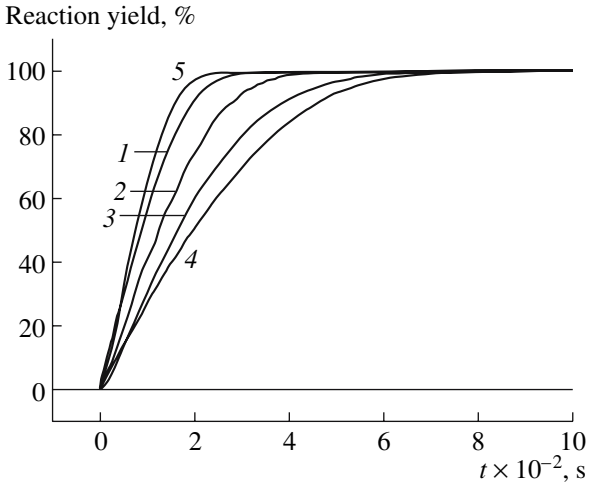

Fig. 1. Typical kinetic curves for the hydrolysis of N-acetyl-L-tyrosine ethyl ester catalyzed by bovine pancreatic α-chymotrypsin preliminary incubated in water–dioxane mixtures with various thermodynamic activities of water: (*1*) 0.01, (*2*) 0.6, (*3*) 0.8, (*4*) 0.9, and (*5*) 1.0.

In the regions of low and moderate water activities, where the enzyme is insoluble, the spectra feature a different pattern. In this case, the binding of proflavin by chymotrypsin gives rise to a minimum in the difference spectra. In these experiments the concentration of the enzyme in the mixture was constant, 1.9×10^{-4} mol/l.

Thermodynamic activity of water in dioxane. The activity of water in dioxane was calculated by the formula

$$a_w = \gamma_w x_w, \quad (1)$$

where x_w is the mole fraction of water in the solution and γ_w is the activity coefficient of water (mole fraction scale; the standard state, pure substance). The water activity coefficients were calculated from the published data on the vapor–liquid equilibrium characteristics for water–dioxane mixtures [13] by the formula

$$\gamma_w = y_w p_t / x_w p_0, \quad (2)$$

where y_w is the mole fraction of water in the gas phase, p_t is the total pressure, p_0 is the saturation vapor pressure over pure water at the same temperature, and x_w is the mole fraction of water in the liquid phase.

RESULTS AND DISCUSSION

Figure 1 shows kinetic curves for the hydrolysis of ATEE catalyzed by a α-chymotrypsin preparation preliminary incubated in water–dioxane mixtures. The catalytic activity was characterized by the ratio of the 200-s reaction yield after incubation of the enzyme in an aqueous organic mixture to that after its incubation in pure water (Fig. 2, curve *1*).

As can be seen from Fig. 2, dioxane influences the catalytic activity of the biocatalyst in a complicated way. At water activities from 0 to 0.5, the catalytic

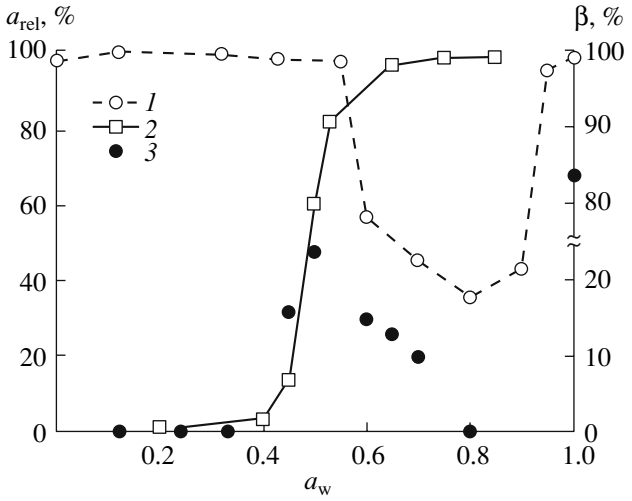

Fig. 2. Relative catalytic activity a_{rel} of α-chymotrypsin in (*1*) the hydrolysis of ATEE and (*2*) solid-phase hydrolysis of N-succinyl-L-phenylalanine *p*-nitroanilide according to [14] and (*3*) the fraction β of bound proflavin as functions of the water activity a_w in an aqueous–organic mixture in which the enzyme was preliminary incubated.

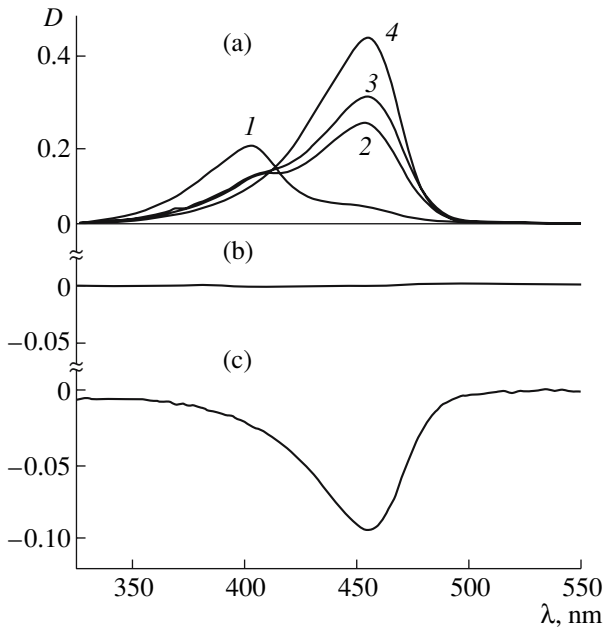

Fig. 3. (a) Typical spectra of proflavin in water–dioxane mixtures with various thermodynamic activities of water ((*1*) 0.12, (*2*) 0.24, (*3*) 0.5, and (*4*) 0.8)) and (b, c) difference spectra of proflavin in the presence of α-chymotrypsin in water–dioxane mixtures at a_w = (b) 0.12 and (c) 0.5. The 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.

activity remains virtually constant, equal to that observed after incubation in pure water. As the water activity increases from 0.5 to 0.9, the catalytic activity decreases sharply. At $a_w > 0.9$ (34 vol % dioxane), the catalytic activity increases, approaching the level corresponding to pure water.

Spectra of proflavin in water–dioxane mixtures. Figure 3a shows spectra of proflavin in water–dioxane mixture. As can be seen, the organic solvent 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 new shortwave absorption band appears, the intensity of which 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–dioxane solution decreases, the equilibrium shifts towards the deprotonated form. This conclusion is supported by the results of the following experiment. Figure 4 displays the spectra of proflavin in water at various pH values. As seen, as in the case of water–dioxane mixtures, variations in the pH 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.

Binding of proflavin in water–dioxane mixtures. Figure 3b shows 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 $a_w = 0.12$ (0.5 vol % water). As can be seen, the presence of the enzyme produces no effect on the proflavin spectrum, which means that, in this mixture, no binding of proflavin by the enzyme occurs. Similar spectra were obtained for mixture with $a_w = 0.24$, 0.33, and 0.8.

Figure 3c 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 $a_w = 0.5$ (3.7 vol % water). Within this range of water activities, α-chymotrypsin is insoluble, and, therefore, the dip in the difference spectra was attributed to a decrease in the concentration of proflavin due to its binding by the enzyme. Similar spectra were obtained for mixtures with $a_w = 0.45$, 0.6, 0.65, and 0.7.

The process of binding of proflavin in water–dioxane mixtures was qualitatively characterized by the absorbance at the isobestic point (at 414 nm, Fig. 4). This was motivated by the following circumstances. On the other hand, it is unknown what form of proflavin, protonated or deprotonated, is bound by the enzyme in aqueous–organic mixtures. On the one hand, at the isobestic point the absorption coefficients of both forms coincide. Correspondingly, by measuring changes in the absorbance at 414 nm, one can determine changes in the concentration of the competitive inhibitor in the solution irrespective of the form in which it is bound. The ratio of the absorbance at the 414-nm point of the

difference spectrum to the absorbance at the isobestic 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 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.

Effect of dioxane on the catalytic activity of α-chymotrypsin and its ability to bind proflavin. The results on the catalytic activity of α-chymotrypsin and its ability to bind proflavin were interpreted within the framework of the model proposed in [1]. According to this model, the dehydration of proteins results in the formation of firm intermolecular contacts via the establishment of hydrogen bonds and/or ionic bridges between side polar groups of the protein (carboxy, alcohol, amido, 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 [1]. Correspondingly, if these groups enter into the composition of the active site of the enzyme, they become incapable of interacting with molecules of the substrate or competitive inhibitor. As a result, α-chymotrypsin shows no catalytic activity in reactions of solid-phase hydrolysis at low water activities ($a_w < 0.4$) in the absence of an organic solvent [14] (Fig. 2).

On the other hand, it was demonstrated that the stability and structure of dry proteins are substantially dependent on the ability of the organic solvent to form hydrogen bonds [15]. Considerable structural changes and exothermic effects were observed only in solvent capable of forming strong hydrogen bonds. Consequently, knowledge of the tradeoff between the proton-donor and proton-acceptor 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 dioxane) molecule is to choose with which fragment to interact, proton-donor or proton-acceptor.

Water molecules can solvate both proton-donor and proton-acceptor groups of the protein. By contrast, proton-acceptor dioxane molecules will predominantly solvate the proton-donor group of the broken contact (the remaining proton-acceptor group will be more effectively solvated by water molecules). Thus, dioxane molecules are incapable of breaking interprotein contacts on their own, in the absence of water. Correspondingly, the interaction of dry α-chymotrypsin with anhydrous dioxane is not accompanied by significant heat effects or structural changes [15]. This means that, at low water activities, dioxane produces no appreciable effect on the state of the enzyme in the form of a low-humidity solid preparation. Therefore, no noticeable decrease in the catalytic activity was observed upon incubation of α-chymotrypsin in the aqueous-organic solvent at $a_w < 0.4$ (Fig. 2).

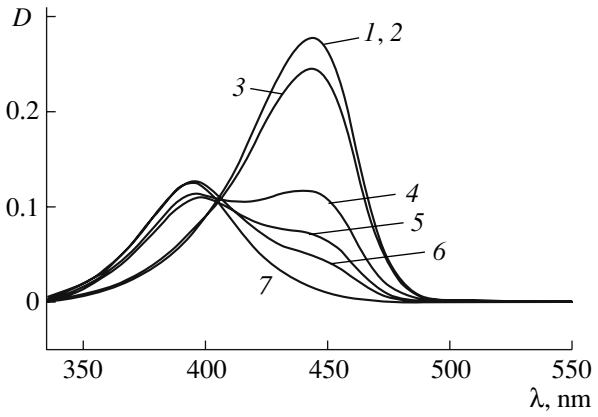

Fig. 4. Typical absorption spectra of proflavin in water at various pH values: (*1*) 3.0, (*2*) 5.9, (*3*) 7.8, (*4*) 8.5, (*5*) 8.9, (*6*) 9.2, and (*7*) 10.5.

Only above the threshold protein humidity ($h \sim 0.1$ g water/g protein 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 [1, 14, 16, 17]. Note that, in our experiments, the significant increase in the degree of binding of the competitive inhibitor and the catalytic activity of the enzyme in the reaction of solid-phase hydrolysis are also observed at $a_w = 0.4$–0.5. According to the proposed model, at low water activities, water molecules penetrate into the structure of the dry 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 (Fig. 2) and its ability to bind proflavin vary in a similar way, both passing through a minimum at $a_w \sim 0.8$. On the one 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 the organic solvent determines the functional characteristics of the enzyme. It was demonstrated [1, Fig. 2] that, in this range of water activities, the enzyme is denaturated by dioxane with the formation of intermolecular β-structures.

At moderate water activities, the degree of binding of proflavin (Fig. 2) passes through its maximum, reflecting the interplay between the following processes. On the other hand, the hydration of the enzyme is already high enough, so that its conformation is close to the native one. On the one hand, some interprotein

contacts remain unbroken, a factor that plays a positive role by hindering the denaturation of the enzyme by the organic solvent.

Note also that, as follows from Fig. 2 and the data reported in [2], the shapes of the isotherms of binding of proflavin by α-chymotrypsin and the positions of the maximum degree of binding in dioxane and acetonitrile are similar. This means that the formation of the enzyme competitive inhibitor complex in moderate-strength proton-acceptor solvents, such as dioxane and acetonitrile, exhibits similar regularities.

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