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THERMODYNAMIC PROPERTIES OF PURE SUBSTANCES

The Influence of Dioxane on the Hydration of Bovine Pancreatic α-Chymotrypsin according to Isothermal Calorimetry and IR Spectroscopy Data

V. A. Sirotkin and D. V. Korolev

Butlerov Chemical Institute, Kazan State University, Kazan, Tatarstan, Russia

E-mail: vsir@mail.ru

Abstract—The influence of dioxane on the thermochemical characteristics of the hydration of bovine pancreatic α-chymotrypsin enzyme over the whole range of water thermodynamic activities was studied by comparing the isothermal calorimetry data on the thermochemistry of interaction between the enzyme and water in the presence and absence of dioxane and using the IR spectral data on the adsorption of water and organic solvent vapors on the protein

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INTRODUCTION

The hydration of protein catalysts (enzymes) is of fundamental importance; it is also of interest for practical applications. It is well known that water plays a key role in biological functioning of enzymes [1, 2]. The interaction of proteins with water is also of importance for the formation of the native spatial structure of proteins.

One of the problems of current interest in this area is the determination of the physicochemical characteristics of the interaction of enzymes with water in the presence of low-molecular-weight compounds, including organic solvents. Solving this problem would be of considerable interest for nonaqueous enzymology and biotechnology. In particular, protein catalysts in organic media can catalyze industrially important synthetic reactions with high regio- and enantioselectivity [3–6]. Thermodynamic equilibria in these reactions are shifted toward synthesis products. The efficiency of such biocatalytic systems, however, depends on the moisture content in the medium substantially.

Understanding the rules that govern the hydration of enzymes in the presence of organic solvents requires the use of effective analytic techniques capable of providing thermodynamic information about enzyme–water and enzyme–organic solvent intermolecular interactions. Infrared (IR) spectroscopy is one of such methods. It has successfully been used to obtain information about the interaction of protein molecules with the environment in aqueous solutions [7, 8] and organic solvents [9–12] and to study the characteristics of hydration of solid protein sorbents [1, 2, 13]. The effectiveness of IR spectroscopy as a tool for studying the competitive adsorption of water and organic solvents on proteins was demonstrated in [14–16].

Isothermal calorimetry has recommended itself as one of the effective methods for obtaining direct thermodynamic information about the special features of interaction of proteins with water in various environments, including organic media. In particular, direct calorimetric measurements of the heat effects of adsorption of water vapor on bovine serum albumin were performed in [17]. The enthalpies of adsorption of water on lysozyme over the range of relative water pressures 0–0.895 were measured calorimetrically in [18].

Isothermal calorimetry was used in [17] to determine the enthalpies of interaction of serum albumin with water in the presence and absence of an organic solvent over the whole range of water thermodynamic activities. A comparative study of the thermochemical characteristics of interaction of serum albumin with aqueous–organic mixtures of various compositions and a study of the adsorption of water on the protein were reported in [19–21]. Thermochemical experiments were performed in eight organic media, including dimethylsulfoxide (DMSO), acetonitrile, dioxane, pyridine, methanol, ethanol, propanol-1, and butanol-1. In all instances, the enthalpy of wetting of the protein was shown to contain substantial contributions of water sorption/desorption on the protein.

In this work, we for the first time made an attempt at quantitatively estimating the influence of an organic solvent on the hydration of enzyme over the whole range of water thermodynamic activities by comparing isothermal calorimetry and IR spectroscopy data. Our goal was to develop an experimental technique for separating the contribution of an organic solvent to the thermochemical and sorption characteristics of hydra-

tion of enzymes over the whole range of water thermodynamic activities.

The object of study was bovine pancreatic α-chymotrypsin. This protein has been studied thoroughly, and its amino acid composition, spatial structure, and mechanism of functioning have been established reliably. It has been widely used to study the behavior of enzymes in both aqueous solutions [22] and organic environments [3–5]. Dioxane was selected as a model organic solvent. It can form strong H-bonds, but, as distinct from water, has no proton donor ability. We used this distinction to analyze the mechanisms of various processes.

EXPERIMENTAL

Materials. Bovine pancreatic chymotrypsin from Sigma, C 4129, was used without purification. The catalytic activity of α-chymotrypsin was 66 units per mg of the enzyme. One unit hydrolyzes 1.0 μmol of ethyl ester of N-benzoyl-L-tyrosine per min at pH 7.8 and 298 K. Dioxane of kh. ch. (chemically pure) grade was purified and dried as recommended in [23] and then held over 3 Å molecular sieves. Doubly distilled water was used.

Isothermal calorimetry. Heat effects were measured at 298 K on a BT-2.15 (Setaram) calorimeter. The instrument was calibrated electrically using the Joule effect. The heat effects of interaction between the protein and aqueous–organic mixtures and solution of the protein in water were measured as follows. A protein sample (5–10 mg) was first loaded into a titanium container with Teflon gaskets. After sealing, the container was placed into the calorimetric cell preliminarily filled with a solvent (4.0 ml). When thermal equilibrium was attained, Teflon gaskets were pierced, and the solvent began to interact with the protein. The equilibrium concentration of water in the liquid phase was determined after calorimetric measurements by electrochemical titration according to Fischer. The procedures for heat effect measurements and electrochemical titration of water are described in detail in [19–21].

Dry protein samples were obtained by holding the initial material at 298 K and pressure 0.1 Pa in an MGDTD-17S microthermoanalyzer (Setaram) until its weight ceased to change. Moisture content in the dry samples was 0.2 ± 0.1% (g water/g protein).

Protein samples with variable moisture content (for measuring the heat effects of protein interaction with water) were obtained by holding the substance in a desiccator at 298 K over saturated aqueous solutions of salts (LiCl, CH_3COOK, $CaCl_2$, K_2CO_3, $Mg(NO_3)_2$, NaCl, $BaCl_2$, and $K_2Cr_2O_7$) and phosphorus pentoxide for 1 week. The activities of water over saturated solutions of the salts were taken from [24]. The moisture content in the protein samples was determined by holding them at 298 K and a pressure of 0.1 Pa in an MGDTD-17S microthermoanalyzer (Setaram) until their weight ceased to change.

IR spectroscopy. The procedure for spectroscopic measurements was described in detail in [14–16]. The IR spectra were recorded on a Vector 22 Fourier transform IR spectrometer (Bruker) at 298 K over the frequency range 4000–1000 cm^{-1} with a 4 cm^{-1} resolution, the number of scans was 256. The samples were optically transparent protein films prepared by vaporizing a 2% aqueous solution of the protein (25 μl) on a CaF_2 cell window in air at room temperature and humidity. The film was placed into a hermetic cell with fluorite windows. The sample was dehydrated directly in the spectrometer by a flow of air dried over phosphorus pentoxide until a constant optical density was established in the region of adsorbed water absorption at 3450 cm^{-1}. The relative water vapor pressure over phosphorus pentoxide at 298 K is lower than 0.01 [25]. The spectrum of this sample was used as a reference to obtain difference spectra. Next, the sample was subjected to the action of pure water or water–dioxane mixture vapors. In the first case, a flow of air was passed first through a saturator with water and then the cell with the protein. The activity of water in the vapor phase was varied by changing the temperature difference between the measuring cell and saturator. The data on water vapor pressures at various temperatures were taken from handbook [24]. In the second case, a flow of dry air was passed through a saturator with an aqueous–organic mixture and the cell with the protein kept at equal temperatures, 298 K. The relative water vapor pressure was varied by changing the activity of water in the liquid aqueous–organic mixture.

The adsorption of water and dioxane was monitored by recording adsorption spectra in the regions of 3450 cm^{-1} for water and 1121 cm^{-1} (the C–O group stretching vibration absorption band) for dioxane. We assumed that protein film swelling caused by water adsorption largely occurred at the expense of an increase in its thickness. The hydration value could then be calculated as

$$h = 2.3S_w\varepsilon_p/B_wD_p, \quad (1)$$

where h is the moisture content in the protein (mol water/mol protein), S_w is the area under the absorption band of water (cm^{-1}), ε_p is the extinction coefficient at the maximum of the amide I band in the spectrum of the protein (l/(mol cm)), B_w is the integral extinction coefficient of water (l/(mol cm)), and D_p is the optical density at the maximum of the amide I band. For water, we used the integral extinction coefficient B_w = 96 000 ± 1000 l/(mol cm) [26]. The sorption of dioxane was calculated by an equation similar to (1). The integral extinction coefficient of dioxane was determined from the area under the pure substance band in a cell 10 μm thick, $B_{dioxane}$ = 19 100 ± 17 l/(mol cm). The extinction coefficient of bovine pancreatic α-chymotrypsin was determined from the spectrum of a solution of the pro-

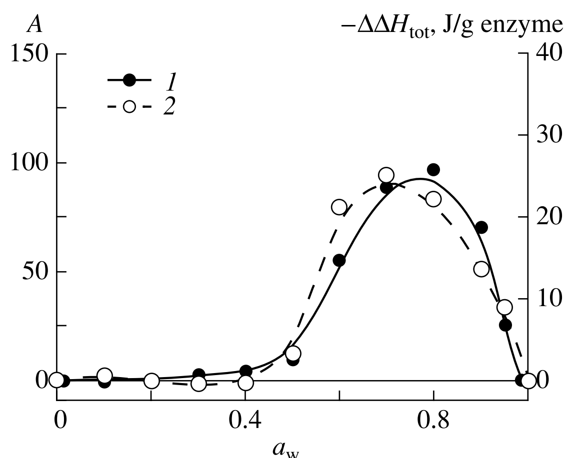

Fig. 1. Adsorption of dioxane vapor (A, mol/mol chymotrypsin) on bovine pancreatic α-chymotrypsin as a function of water activity. The differences in the heat effects ($\Delta\Delta H_{tot}$) of interaction between chymotrypsin and water in the presence and absence of the organic solvent.

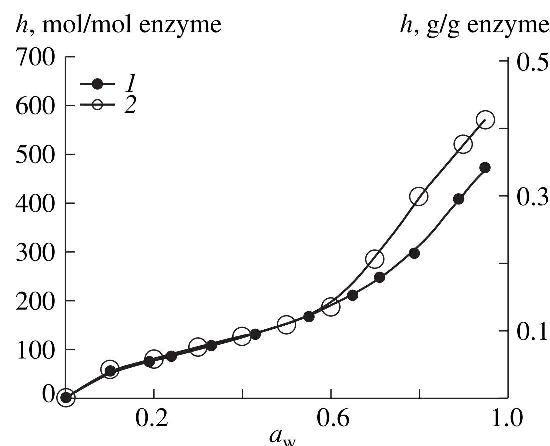

Fig. 2. Isotherms of water vapor adsorption (a_w) on α-chymotrypsin: (*1*) adsorption of water in the absence of dioxane vapor, and (*2*) adsorption of water in the presence of dioxane vapor. The solid lines are polynomial approximations of adsorption isotherms. Errors in the experimental moisture contents are of 0.002–0.005 g water/g protein.

tein in heavy water of a certain concentration, ε_p = 80000 ± 700 l/(mol cm). The molecular weight of the protein was taken to be 25000.

The activity of water in dioxane. The activity of water in dioxane was calculated by the equation

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

where x_w is the mole fraction of water in the liquid phase and γ_w is the activity coefficient of water (mole fraction scale, pure water as the standard state). The activity coefficients of water were calculated from the literature data on vapor–liquid equilibrium for water/dioxane mixtures [27] by the equation

$$\gamma_w = y_w p_t / x_w p_0, \quad (3)$$

where y_w is the mole fraction of water in the gas phase, p_t is the total pressure, p_0 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.

RESULTS AND DISCUSSION

Adsorption of Dioxane and Water

The isotherm of dioxane vapor adsorption from binary aqueous–organic mixtures is shown in Fig. 1 (curve *1*). The initial state for studying the adsorption of dioxane was a protein sample dried in a flow of air at water activity lower than 0.01. Figure 1 shows that the isotherm of dioxane adsorption contains two portions. At low water activities, dioxane is virtually not adsorbed on the enzyme. At a water activity of about 0.5, the adsorption of the organic substance sharply increases and reaches a maximum at a_w = 0.7–0.8. At water activities higher than 0.8, the adsorption of dioxane sharply drops to zero.

The isotherms of water vapor adsorption on chymotrypsin from both pure liquid water and water–dioxane mixtures are shown in Fig. 2. The initial state for studying the adsorption of water was a protein sample dried in a flow of air at water activity lower than 0.01, as with dioxane. All the water adsorption isotherms had a characteristic S shape, which was in close agreement with the literature data [1, 15, 16, 28].

The presence of organic compound vapor noticeably influences the ability of the enzyme to adsorb water. The influence of dioxane on the adsorption of water was characterized by the difference in water adsorption (Δh) between the corresponding branches of adsorption isotherms (Fig. 3). Two different organic solvent effects on the adsorption of water by the enzyme were observed. At low water activities (≤0.5), the adsorption isotherms were close to each other, which was evidence of the absence of a noticeable influence of the organic solvent on sorption. This finding is in agreement with the results obtained in [15, 29]. At water activities higher than 0.5, the isotherm of water adsorption measured in the presence of dioxane vapor lies above the corresponding branch for pure water vapor. This is evidence of activation of water sorption by the enzyme under the action of dioxane.

The Heat Effects of Water Interaction with Chymotrypsin in the Presence and Absence of Dioxane

The heat effects ΔH_{tot} of chymotrypsin interaction with water in the presence and absence of the organic solvent were determined as follows. *The initial state* was the protein dried in a flow of air at a water activity of 0.01 and 298 K. *The final state* was the protein in an aqueous–organic mixture with a variable water activity

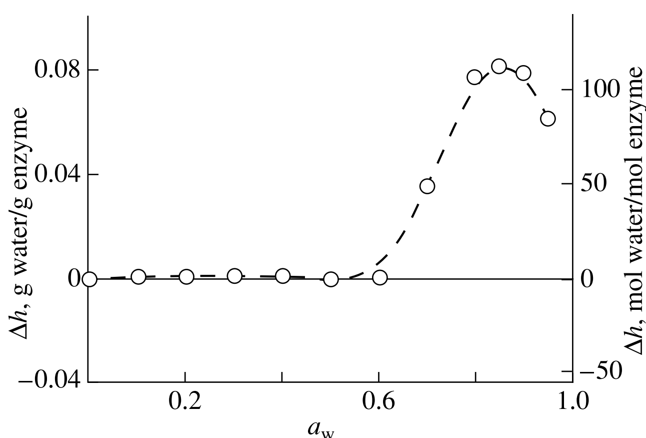

Fig. 3. Difference in the hydration of bovine pancreatic α-chymotrypsin (Δ*h*) between water adsorption isotherms in the presence and absence of dioxane vapor.

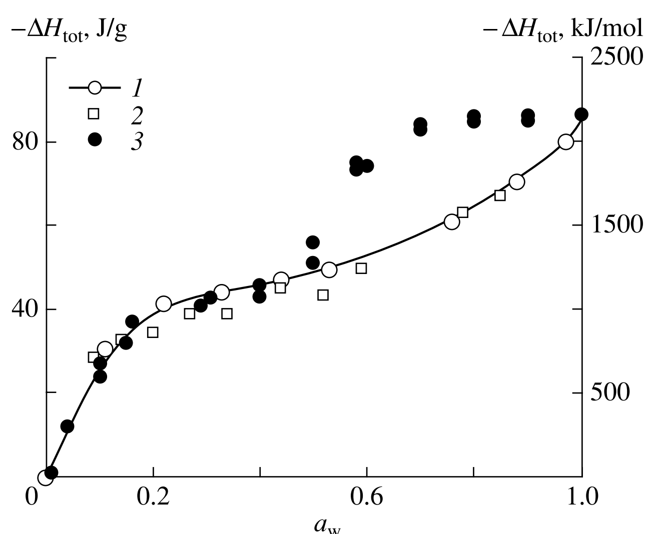

Fig. 4. Heat effects (ΔH_{tot}) of interaction of bovine pancreatic α-chymotrypsin with water in the absence ((*1*) data from [30] and (*2*) this work) and (*3*) presence (this work) of dioxane.

or the protein at equilibrium with water vapor with a variable activity transferred from pure liquid water at 298 K and 1.01×10^5 Pa.

The ΔH_{tot} (kJ/mol protein) value is the difference of the partial molar enthalpies of the protein in the system with a variable water activity ($\overline{H}_p$) and the protein in the dry state ($\overline{H}^\circ_p$),

$$\Delta H_{tot} = \overline{H}_p - \overline{H}^\circ_p. \quad (4)$$

Calorimetric data of two types were used to determine ΔH_{tot} in the absence of dioxane.

Heat effects of solution of moistened chymotrypsin samples (ΔH_{sol}) and the corresponding moisture content values in protein samples held over saturated solutions of salts at 298 K

No.	Salt	a_w	*h*, %	$-\Delta H_{sol}$, kJ/mol protein
1	LiCl	0.11	3.8 (0.4)	1253 (78)
2	CH_3COOK	0.22	5.7 (0.5)	984 (75)
3	$CaCl_2$	0.33	7.5 (0.8)	910 (71)
4	K_2CO_3	0.44	9.7 (0.8)	838 (62)
5	$Mg(NO_3)_2$	0.53	11.8 (0.9)	784 (61)
6	NaCl	0.75	20.0 (1.2)	500 (45)
7	$BaCl_2$	0.88	28.6 (2.5)	275 (14)
8	$K_2Cr_2O_7$	0.97	36.0 (3.7)	25 (12)

Note: The standard root-mean-square deviations are given in parentheses; each value was obtained in three or four measurements; *h* is the moisture content in protein samples (g/g protein).

(1) The ΔH_{tot} values (Fig. 4, data set no. 1) were calculated by the equation

$$\Delta H_{tot} = (\Delta H_{hydr} + \Delta H_{vap})h, \quad (5)$$

where ΔH_{hydr} is the enthalpy of water vapor adsorption on the protein (kJ/mol water) [24], ΔH_{vap} is the enthalpy of vaporization of water (43.7 kJ/mol water [24]), and *h* is the moisture content in the protein (mol water/mol protein). We used the enthalpies of adsorption of water vapor on chymotrypsin from [30]. The ΔH_{hydr} values were calculated from the temperature dependence of the isotherms of water vapor adsorption on chymotrypsin [30].

(2) The ΔH_{tot} values were determined in this work (Fig. 4, data set no. 2) from isothermal calorimetry data by the equation

$$\Delta H_{tot} = \Delta H^\circ_{sol} - \Delta H_{sol}, \quad (6)$$

where ΔH°_{sol} is the enthalpy of solution of dry protein in water at 298 K (kJ/mol protein) (according to [12], $\Delta H^\circ_{sol} = -2165 \pm 70$ kJ/mol chymotrypsin, or -86.6 ± 2.8 J/g chymotrypsin) and ΔH_{sol} (kJ/mol protein) is the enthalpy of solution in water of the protein moistened by holding it over saturated aqueous solutions of salts (table).

The dependence of the heat effects of interaction of dry chymotrypsin with water–dioxane mixtures on water activity is shown in Fig. 4 (data set no. 3). The influence of dioxane on ΔH_{tot} was characterized by the difference thermochemical values (Fig. 1, curve *2*, $\Delta\Delta H_{tot}$). Two regions can be distinguished in Fig. 1, curve *2*. At water activities below 0.5, dioxane does not

influence the thermochemical characteristics of protein hydration. At high water activities, the difference thermochemical values are essentially nonzero.

The thermochemical results are in close agreement with the IR data on dioxane and water adsorption. According to Fig. 1 (curve *1*), the adsorption of dioxane is close to zero at low water activities. The influence of dioxane on the hydration of the protein and its thermochemical properties is also insignificant (Fig. 3 and Fig. 1, curve *2)*. The organic solvent substantially influences the thermochemical and sorption properties of the protein at high water activities only.

The Influence of Dioxane on the Thermochemical and Sorption Properties of α-Chymotrypsin

Water activity below 0.5. The influence of dioxane on the adsorption of water by the protein can be explained on the basis of the hypothesis of the existence of strong intermolecular contacts in dry protein samples. According to this hypothesis, which was put forward in [15, 29], the dehydration of proteins is accompanied by the formation of intermolecular contacts, predominantly H-bonds and/or ionic bridges between protein polar groups. This results in the formation of a protein structure with an increased rigidity, however, noticeably distorted compared with the native structure. A substantial fraction of polar groups in dry proteins then becomes incapable of acting as adsorption centers because they already participate in the formation of interprotein contacts.

On the other hand, it was shown that the stability and structure of dry proteins depend substantially on the ability of organic solvents to form H-bonds [12]. Considerable structural changes and exothermic heat effects were only observed if solvents (e.g., DMSO) were capable of forming strong H-bonds. It follows that knowledge of the balance of the proton donor and proton acceptor properties of solvents is of importance for predicting possible organic molecule effects on the sorption properties of protein sorbents. For instance, when an interprotein contact such as an H-bond gets broken, sorbate molecules (water or dioxane) can select between the proton donor and proton acceptor broken contact fragments. Water molecules solvate both proton donor and proton acceptor protein groups. Proton acceptor dioxane molecules in turn only solvate broken contact proton donor groups, whereas water molecules more effectively solvate the remaining proton acceptor groups. It follows that dioxane molecules are incapable of breaking interprotein contacts by themselves, in the absence of water. Nor can dioxane molecules be sorbed on dry proteins simultaneously with water, likely because of steric and diffusion limitations. The molar volume of dioxane (85.2 cm^3/mol) is more than four times larger than that of water (18 cm^3/mol). For this reason, we do not observe noticeable dioxane adsorption at the lowest water activities (Fig. 1, curve *1*). No noticeable thermal effects and structural changes were also observed when dry chymotrypsin was introduced into water-free dioxane (Fig. 1, curve *2*, and Fig. 4) [12].

It follows that the main process that occurs at water activities of from 0 to 0.5 is the interaction of water with the protein. According to the suggested model, sorption then occurs as the insertion of water molecules into the structure of dry sorbent samples, rupture of interprotein contacts, hydration of the polar groups of these contacts, and formation of new sorption sites.

Water activity above 0.5. According to the current concepts [1, 2], the hydration of protein polar groups is completed in this region in the absence of organic solvents. Next, water interacts with weakly bonding protein macromolecule regions with the formation of a multilayer coating. Eventually, the spatial native protein structure is formed.

Noticeable differences in the mechanism of enzyme hydration are observed in the presence of organic solvents. For instance, above $a_w = 0.5$, the rupture and hydration of the polar fragments of the majority of interprotein contacts are followed by a sharp increase in dioxane sorption. Note that each dioxane molecule contains four hydrophobic methylene groups. We can therefore assume that, when inserted into the structure of a hydrated protein sorbent, dioxane (unlike water) can solvate polar groups not only in hydrophilic but also in hydrophobic protein molecule regions with the formation of new sorption sites for water. An additional amount of adsorbed water (compared with the sorption of pure water) at $a_w > 0.5$ (Fig. 3) is an example of the activating action of dioxane on the sorption of water on the protein. Interestingly, the largest number of excessively sorbed water molecules (Fig. 3) is close to the number of dioxane molecules adsorbed at $a_w = 0.8$ (~100 mol/mol enzyme) (Fig. 1, curve *1*).

The difference thermochemical characteristics are also sensitive to changes in the mechanism of water adsorption (Fig. 1, curve *2*). When serum albumin is introduced into organic solvents with bulky hydrophobic groups (pyridine, butanol-1, and propanol-1), substantial exothermic jumps in the enthalpies of wetting are observed at medium water activities (~0.5–0.6) [20] (table).

To summarize, according to the suggested model, the special feature of enzyme hydration at high water activities is the insertion of organic solvent molecules predominantly into hydrophobic protein sorbent regions, rupture of interprotein contacts, and creation of new sorption sites. The maxima of dioxane sorption (Fig. 1, curve *1*), the excessive amount of water adsorbed (compared with the adsorption of pure water) (Fig. 1, curve *2*), and the $\Delta\Delta H_{tot}$ values at $a_w = 0.8$ (Fig. 1, curve *2*) are evidence in favor of this adsorption mechanism.

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