CALORIMETRIC AND FTIR-SPECTROSCOPIC STUDY OF SOLVENT EFFECT ON THE STATE OF DRY SOLID BOVINE PANCREATIC α-CHYMOTRYPSIN IMMERSED IN ANHYDROUS ORGANIC SOLVENTS

V. A. Sirotkin*, A. N. Zinatullin*, B. N. Solomonov*, D. A. Faizullin, and V. D. Fedotov****

Calorimetric heat effects and structural rearrangements accompanying the immersion of dry solid bovine pancreatic α-chymotrypsin in anhydrous organic solvents and water were measured at 298 K. It was found that the enthalpy and IR-absorbance changes being put together obey good linear correlation. According to the extent of their influence on the protein structure and thermodynamic state the solvents could be divided into two groups. The first group exhibiting nearly zero effects consists of carbon tetrachloride, benzene, nitromethane, acetonitrile, 1,4-dioxane, n-butanol, n-propanol and pyridine. Dry solid protein is suggested to be stable in such media due to kinetic reasons. Immersion of the protein into a second group solvents, namely, dimethyl sulfoxide, methanol, ethanol, and in pure water as well, is followed by swelling of the protein and accompanied with significant exothermic enthalpy change and structural rearrangements. It was shown that attribution of the solvent to the first or the second group is determined by its thermodynamic hydrophilicity (partial excess molar Gibbs free energy of water in a given solvent at infinite dilution). The first group consists of liquids with thermodynamic hydrophilicities all above 2.7 kJ/mol. The thermodynamic hydrophilicities of the second group solvents are lower than 2.3 kJ/mol. At close hydrophilicities the presence of mobile protons in the solvent molecule sufficiently accelerates the solid protein swelling. It is deduced that thermodynamic hydrophilicity and proton donating ability could be principal factors controlling the stability of dry solid proteins and kinetics of swelling in liquids examined at room temperature.

Introduction

It has been firmly established that the solid proteins immersed in organic solvents with low water content exhibit some remarkable properties—ability to catalyse reactions not feasible in aqueous media [1, 2], greatly enhanced thermostability [3], "molecular memory" [4]. This powerful biotechnological potential of suspended proteins essentially depends on the nature of organic solvent (activating enzymes by denaturing cosolvents [5], affecting the enantioselectivity of suspended enzymes [6], demonstrating exothermic peaks on DSC-curves for human serum albumin immersed in hydrophilic liquids [7, 8], lowering the imprinted protein—ligand binding with increasing the solvent's propensity to form hydrogen bonds [4]).

Therefore direct information on the thermodynamics of the solid protein - organic solvent interactions and structure of its constituents is of importance in explaining various protein activities. Due to ability to monitor the thermodynamic and structural characteristics of processes in such heterogeneous systems the calorimetric methods and IR-spectroscopy have a great potential in understanding factors governing the solid protein - solvent interactions.

Earlier we proposed a calorimetric approach to the investigation of intermolecular processes that occur on immersing the solid proteins in water-organic mixtures [9, 10]. This approach involved the measurement of the enthalpies corresponding to the formation of the "protein + liquid" heterogeneous systems. It was established [9, 10] that the enthalpy of formation of partially hydrated protein suspensions in water-organic mixtures is determined mainly by two processes. The first of them is the water desorption/sorption. Solvent effect on thermodynamics of water sorption by the protein strongly correlates with the water solvation thermodynamics in organic media [9]. The second process is followed by the significant heat effect and increase in the surface area accessible for sorption of water. It was qualified as protein swelling in water-organic mixture [7, 9]. But it is remained unclear whether the processes are accompanied by conformational changes and of what kind they could be.

On the other hand FTIR spectroscopy has been successfully applied to investigation of the state of protein secondary structure in organic solvents of different nature [11, 12]. It was shown that several solvents could induce conformational changes in protein while others are not but still remains unknown what are the factors governing the protein structure in low-water organic media.

Proceeding from the above considerations in the present study we attempted to elucidate (a) what are the relationships between calorimetric and structural parameters of protein state and (b) what is the effect of solvent nature

* Kazan State University, Kremlevskaya str., 18, Kazan, 420008, Russia. E-mail: vsir@mail.ru.
** Kazan Institute of Biochemistry and Biophysics, Kazan, 420503, Russia.

on protein secondary structure and heat effects of interaction of solid protein with organic liquids. Bovine pancreatic α-chymotrypsin was used as model protein.

As is known the water sorption on the protein suspended in organic solvents significantly influence catalytic properties [2], conformation [12], thermostability [3, 8] and calorimetric enthalpies of suspension formation [9, 10]. It seems rather difficult at present time to rationalise separately protein—water and protein—organic solvent interactions in such complex systems. Therefore the starting point of calorimetric and spectral experiments was the essentially dry solid protein (dehydrated at water activity no more than 0.01) plus anhydrous pure organic solvents.

The data obtained were then compared with those characterising the protein state in water, its natural environment. Therefore organic solvents were selected as to compose ascending series on their hydrophilicity.

1. Materials and Methods

Materials. Bovine pancreatic α-chymotrypsin (EC 3.4.21.1; specific activity of 52 units/mg of solid) were obtained from Sigma Chemical Co. (Product No. C 4129). Organic solvents were purified and dried according to the recommendations [13] and than were stored over dry 3A molecular sieves for at least 24 h prior to use.

FTIR measurements. FTIR-spectrometry has been carried out on Vector 22 (Bruker) FTIR-spectrophotometer at 4 cm^{-1} resolution. Vibration spectra were obtained with a glassy like protein films casted from 2% (w/v) water solution onto the CaF_2 window at room humidity. After mounting windows in the sample cell the film was dehydrated flushing the stream of air dried over P_2O_5 powder. Relative pressure of water vapors over phosphorus pentaoxide at 298 K does not exceed 0.01 [14]. The sample was flushed until no further spectral changes were detected in 3450 cm^{-1} water absorbance region and amide A contour in this side represented a smooth line without any visible shoulders. The cell was then filled by the desired organic solvent and a number of spectra were recorded as a function of time until equilibrium was achieved. For to compensate solvent absorbance the spectra from another cell of equal gap filled by the same organic liquid but without protein sample were recorded and afterwards digitally subtracted from sample spectra.

Protein spectra in water environment have been obtained on highly hydrated films by flushing damp air at 99% relative humidity. Spectrum of liquid water was then digitally subtracted from the spectra of wet films in accord with the criteria described in Ref. [15]. Secondary structure alterations have been analyzed using known correlation between secondary structure elements in proteins and peak positions in the amide I spectra [16, 17].

Calorimetric measurements. Commercial lyophilized protein preparation was further dried under vacuum using microthermoanalyzer equipment (Setaram, MGDTD-17S) at 298 K and 0.1 Pa until approaching constant sample weight. Water content of the dried protein was estimated as 0.003 ± 0.003 g of water/g of dry protein by the Karl Fischer titration method [18]. Calorimetric heat effects on immersing the dry protein powder into organic solvents and water were measured at 298 K with a Setaram BT-215 microcalorimeter according to the described procedure [10]. Typically, 4–8 mg of protein sample was placed in the calorimetric cell and brought in contact with 4.0 ml of a given solvent. The enthalpy changes on dissolution of the dry CT powder in water were measured at protein concentration of 1 g/L.

Solubility. The dry solid α-chymotrypsin were insoluble in all the studied organic solvents as confirmed by measurements on Vector-22 FTIR-spectrophotometer in amide I region. No noticeable variation in the absorbance of the liquid phase was observed after exposing the protein sample for at least 6 h to the studied anhydrous organic solvents.

Solvent hydrophilicity. Partial molar excess Gibbs free energy $\overline{G}^{E\infty}$ of water in a solvent at infinite dilution and 298 K was used as a measure of solvent hydrophilicity. The $\overline{G}^{E\infty}$ values have been calculated by us in Ref. [19] using the equation

$$\overline{G}^{E\infty} = RT \ln \gamma_w^{\infty}, \qquad (1)$$

where γ_w^{∞} is a mole fraction basis activity coefficient for water at infinite dilution. ($\gamma_w \to 1$ at $x_w \to 1$). Reference state is pure liquid water. In such a hydrophilicity scale the more hydrophilic solvent holds the more negative $\overline{G}^{E\infty}$ value. Partial molar excess Gibbs energy of water in water itself equals zero. The $\overline{G}^{E\infty}$ values are presented in Table 1.

Table 1

Enthalpy changes (ΔH_{tot}) on immersing the dry solid chymotrypsin into anhydrous organic solvents and water, corresponding normalized areas (ΔA) of positive parts in solvent induced difference spectra and the partial molar excess Gibbs free energy ($\overline{G}^{E\infty}$) of water at infinite dilution in the solvent at 298 K

No.	Solvent	ΔH_{tot} (J/g)	ΔA (cm^{-1})	$\overline{G}^{E\infty}$ (kJ/mol)
First group				
1	Carbon tetrachloride	1.6 ± 2.5	0.2 ± 0.1	18.5
2	Benzene	—	0.4 ± 0.4	15.0
3	Nitromethane	1.8 ± 0.2	—	9.0
4	Acetonitrile	9.4 ± 2.8	0.7 ± 0.5	5.0
5	1,4-Dioxane	2.8 ± 2.3	0.7 ± 0.4	4.6
6	*n*-Butanol	—	0.5 ± 0.3	4.2
7	*n*-Propanol	—	0.8 ± 0.3	3.4
8	Pyridin	-0.4 ± 1.1	0.7 ± 0.3	2.7
Second group				
9	Ethanol	-38.1 ± 1.9	4.2 ± 0.7	2.3
10	Methanol	-63.0 ± 2.5	4.4 ± 0.7	1.0
11	Water	-86.6 ± 2.1	8.5 ± 1.5	0
12	DMSO	-76.3 ± 2.1	6.8 ± 0.8	-3.0

The data are presented as the average ± S.E. for 3–6 independent determinations.

2. Results and Discussion

Enthalpy and integral absorbance change following immersing of dry solid chymotrypsin in anhydrous organic solvents. Figure 1*a* represent CT

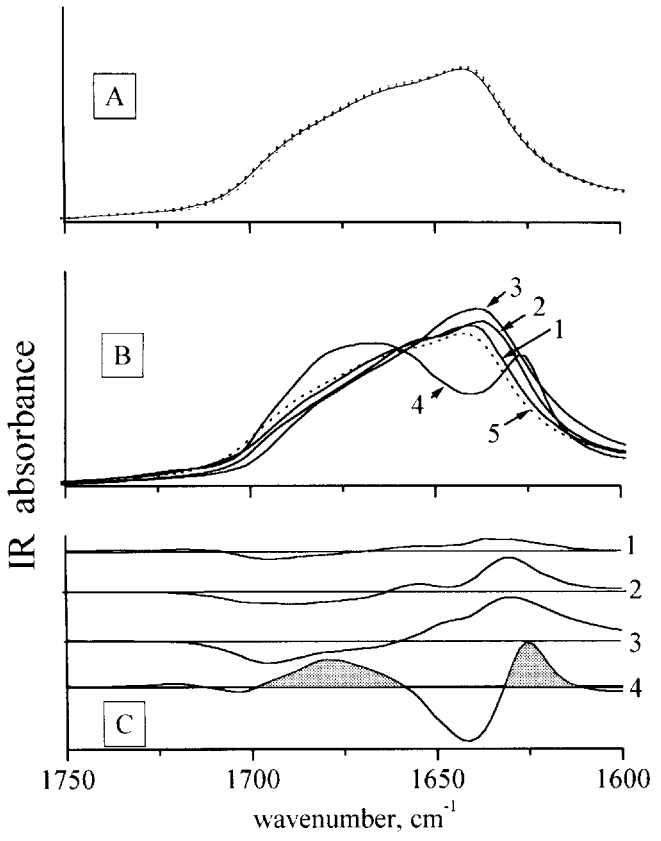

Fig. 1. Amide I spectra of dry chymotrypsin films immersed in (A) first group solvents (solid line—in dry air, dashed lines—in anhydrous organic solvents: carbon tetrachloride, benzene, acetonitrile, 1,4-dioxane, *n*-butanol, *n*-propanol, and pyridine) and (B) second group solvents (*1*—in ethanol, *2*—in methanol, *3*—in water, *4*—in DMSO, and *5*—in dry air). (C) difference spectra, induced in the second group solvents (respective lines *1–4* were shifted for clarity). As example the calculated positive area (ΔA) of difference spectrum in DMSO is shown shaded.

spectra in the carbon tetrachloride, benzene, acetonitrile, 1,4-dioxane, *n*-butanol, *n*-propanol and pyridine. Only subtle absorbance changes if any could be recognized in this case. Figure 1*b* reveals much more sizable alterations when immersing dry protein in methanol, ethanol, DMSO and water.

The overall changes in the protein secondary structure on immersing into organic solvents or in water environment relative to its initial state in the dry solid sample were quantitatively characterized by the positive area of solvent induced difference spectrum of protein in the amide I region (ΔA). The corresponding areas induced for example by DMSO are shown shaded in Fig. 1*c*. All ΔA values are normalized on amide I peak absorbance of the protein sample in dry air.

Corresponding ΔA values, enthalpies of the formation of heterogeneous "solid enzyme + organic solvent" systems and enthalpy changes on dissolution of the dry CT powder in water ($\Delta H_{\rm tot}$) are presented in Table 1.

Obviously interaction of the dry protein with its environment depends on a variety of factors therefore measured calorimetric heats and spectral alterations are those of complex nature. Nevertheless this bulk parameters being put together obey linear correlation both for CT,

$$-\Delta H_{\rm tot} = (-6.9 \pm 6.3) + (12.2 \pm 1.3)\Delta A, \qquad (2)$$

correlation coefficient $r = 0.972$, rms deviation $s_0 = 10.5$, number of points $n = 7$.

Nearly zero constant term in Eq. (2) means that calorimetric heat effects reflect predominantly the structural rearrangements. On the other hand observed linear dependences $\Delta H_{\rm tot}$ on ΔA values suggest that the more structure has been changed the proportionally more heat has been emitted irrespective of the solvent nature and particular secondary structure rearrangements.

Influence of solvent nature on enthalpy and integral absorbance changes. The data of Table 1 were analyzed to find correlation between measured calorimetric and spectral changes, on the one hand, and the most widely used solvent properties, namely, polarity, hydrophobicity, molecular size, Lewis base, and acid strength as well as thermodynamic hydrophilicity, on the other. Dielectric constant ε [20], $\log P$ [21] (where P is partition coefficient between 1-octanol and water), molar volume V_m of solvent molecule, donor and acceptor numbers [20] and the partial molar excess Gibbs energy $\overline{G}^{E\infty}$ of water in a given solvent at infinite dilution (Table 1) were used to characterize the outlined properties of solvents.

ΔH and ΔA values for chymotrypsin show no obvious correlation with the solvent dielectric constant, hydrophobicity, molar volume, donor number and acceptor number (Fig. 2*a*–*e*). Observing the presented data sets one could easily find that in some cases to the given certain argument value more than one single value of $\Delta H_{\rm tot}$ (and ΔA as well) function corresponds simultaneously. Those are points 3, 4, and 10 in Fig. 2*a*; 4 and 9 in Fig. 2*b*; 3, 4, and 9 in Fig. 2*c*; points 7, 9, 10, and 11 in Fig. 2*d*; 6, 7, and 9 or 3, 4, and 12 in Fig. 2*e*. Observed discrepancy between measured $\Delta H_{\rm tot}$ (or ΔA) values for named points is hardly to be explained without additional suggestions.

But only along the thermodynamic hydrophilicity the data turns out to be arranged unequivocally (Fig. 2*f* and Table 1). The solvents examined could be definitely shared between two groups in accord with the values of calorimetric heats and structural alterations they induce in dry protein and the attribution of the solvent to the first or the second group is determined completely by its thermodynamic hydrophilicity.

The first group consists of liquids of positive thermodynamic hydrophilicity values above 2.7 kJ/mol: carbon tetrachloride, benzene, nitromethane, acetonitrile, 1,4-dioxane, *n*-butanol, *n*-propanol, and pyridine. The enthalpies of interaction of the first group solvents with dry solid protein are close to zero. The second group consists of the most hydrophilic liquids: water, DMSO, methanol and ethanol. Their $\overline{G}^{E\infty}$ values are lower than 2.3 kJ/mol. Immersion of dry solid albumin into the second group solvents is followed by significant exothermic enthalpy changes. Heat effects of water and DMSO interactions with solid chymotrypsin are of the most exothermic ones.

According to the extent of structural changes induced in dry solid protein the solvents can be divided into the same two groups. In the solvents of first group the protein

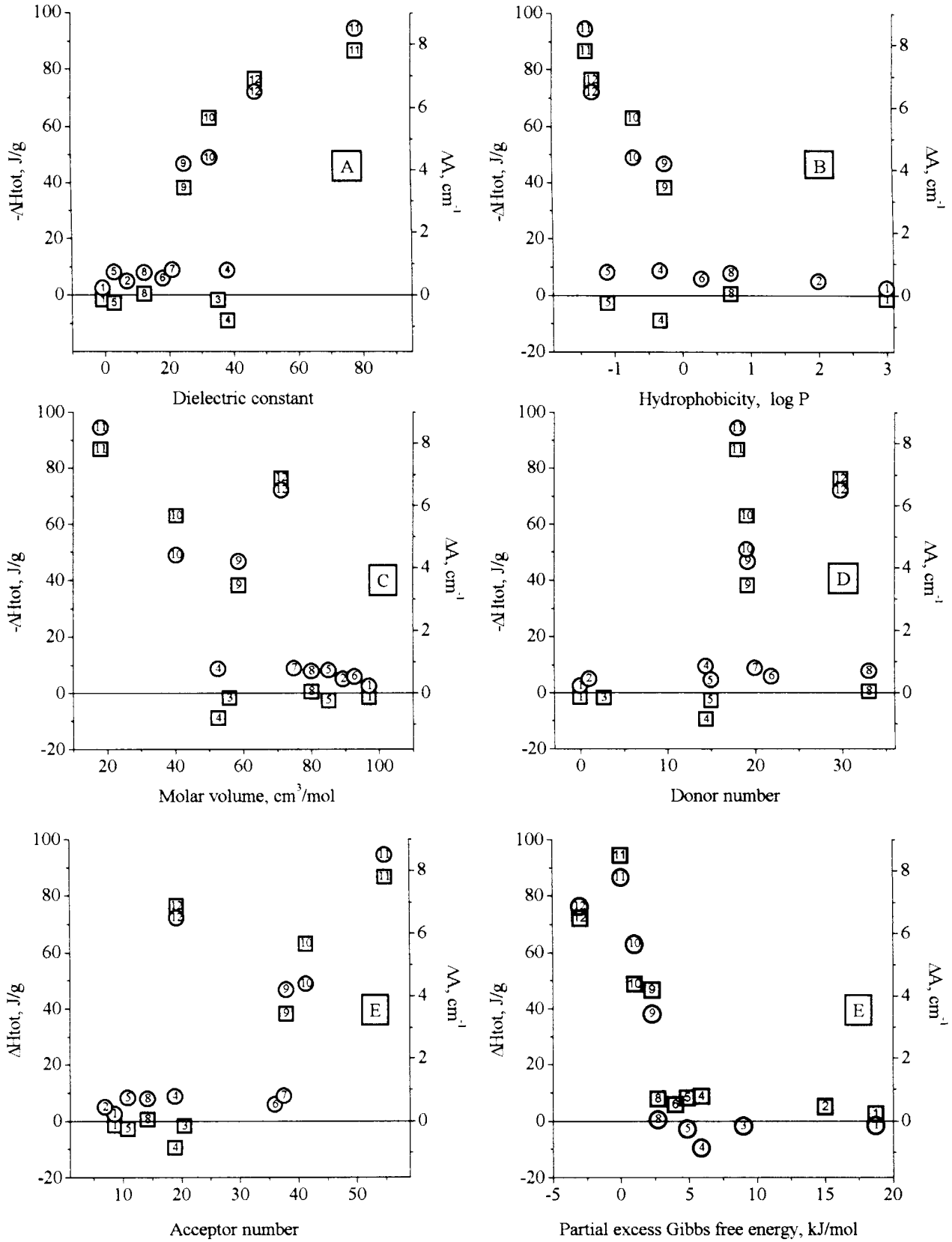

Fig. 2. Enthalpy changes (ΔH) and positive areas (ΔA) in difference spectra induced by organic solvents in dry chymotrypsin plotted vs solvent's dielectric constant (A), hydrophobicity (B), molar volume (C), donor number (D) and acceptor number (E) and partial excess molar Gibbs free energy of water at infinite dilution in a given solvent at 298 K (F). Squares—ΔH values, circles—ΔA values.

secondary structure does not differ markedly from its initial state in dry CT in the absence of solvent ($\Delta A \cong 0$). Immersion of the dry protein into the second group solvents is followed by essential changes in protein structure ($\Delta A > 4.0$ cm^{-1}). The most significant changes are observed in water and in DMSO.

We believe that interaction of dry solid protein with solvents of the second group should not be considered as a simple physical adsorption on surface of solid protein phase. This process if took place has not to induce significant changes in the structure of sorbent. It is more probable that both the essential changes in the protein secondary structure and exothermic heat effects observed in the second group solvents manifest the swelling of dry solid biopolymer in organic low molecular liquids (i. e., dissolution of the organic molecules in solid protein phase). In case of water the protein swelling is followed by dissolution.

The dry solid protein keeps to be stable against the first group solvents at room temperature regardless of proton donating ability of solvent, polarity, hydrophobicity, molecular size, Lewis base and acid strength, as well as thermodynamic hydrophilicity. The reason is that the protein being dehydrated falls in kinetically "frozen" state [22]. The energy barrier of solid protein swelling seems to remain too high in this group of solvents to be overcome at room temperature. This conclusion is supported by results of Ref. [7] where the thermostability of HSA suspension in *n*-hexane-pyridine mixtures has been examined by DSC. It was found that solid HSA suspended in pyridine (a solvent belonged to the first group) at 298 K is in the non-equilibrium state which is evidenced by appearance of exothermic peak on the DSC curves at higher temperatures.

Influence of proton donating ability. The proton donating ability of solvent molecule seems to be the crucial factor lowering potential barrier of swelling of solid protein. For example, thermodynamic hydrophilicities of EtOH and pyridine are close (2.3 and 2.7 kJ/mol, respectively). At the same time the ΔA and ΔH_{tot} values are nearly zero for both proteins in proton accepting pyridine, but immersing dry protein into proton donating ethanol is followed by the significant structure and heat effects (Table 1). Only on heating of the HSA suspension in pyridine to about 353 K did the DSC-thermogram exhibit exothermic peak due to the protein swelling (-34 J/g) [7].

As an another example confirming the influence of proton donating ability of the solvent on the potential energy barrier of solid protein swelling the following pair of solvents may be considered such as water and DMSO. Among the liquids examined those are the most hydrophilic solvents. Their $\overline{G}^{E\infty}$ values equal to 0 and -3 kJ/mol, respectively. But the heat evolution following immersing the dry CT in water was completed within 30 min and in contrary continued to slowly exude during about 2 h in proton acceptor DMSO. Analogous extended heat evolution on immersing the HSA preparation containing 0.1 g of water/g of dry protein into water–DMSO mixtures was observed by us earlier [23]. The same trends have been registered in spectral measurements.

In our mind, it is reasonable to point to the following: among the proton accepting solvents examined just only one—DMSO—was able to induce structure alterations in dry protein. But even in DMSO the rate of structure rearrangements turns out to be much slower than in the less hydrophilic methanol and ethanol (time course of heat evolution within 40 min). It may be deduced that in proton donating media the height of energy barrier of swelling is lower than in proton accepting ones of nearly equal thermodynamic hydrophilicities.

Classification of solvent effect on protein swelling. Proceeding from the above considerations we propose a quality scheme that could allow to predict the behavior of dry solid protein in organic solvents on the base of their thermodynamic hydrophilicity and proton donating ability. The scheme depicts the potential energy changes of reacting features along the reaction coordinate for a three typical cases presented in the this work. The primary idea the scheme is founded on is that the potential energy barrier of protein swelling depends mainly on hydrogen bonding between protein and solvent. According to this idea the more hydrogen bonds contribute to the total energy of formation of the "solid protein + organic solvent" system the lower energy barrier of swelling is observed and protein swelling became more probable.

From the other hand the $\overline{G}^{E\infty}$ value is a measure of the deviation of water state in organic solvent from ideality [24]. Obviously this deviation mainly arises from the formation of hydrogen bonds between water and organic molecules. Consequently the $\overline{G}^{E\infty}$ values may be considered as good characteristics of the degree of such hydrogen bonding. This means that the more negative $\overline{G}^{E\infty}$ value is observed the more significant contribution from hydrogen bonding could be expected in a given solvent. Therefore we suggest that among the solvents examined the energy contribution from hydrogen bonding between protein and organic solvent to the total energy of system formation changes proportionally to the thermodynamic hydrophilicity. Concerning the data reported it means that in the first group solvents where the value of thermodynamic hydrophilicity exceeds 2.7 kJ/mol the contribution from hydrogen bonding is too small and potential barrier of solid protein swelling is too high to be overcome at room temperature. Only at heating to essentially high temperatures the pool of energetically rich molecules becomes large enough for they to jump over the barrier and reaction to proceed markedly—just the situation observed for dry HSA suspended in pyridine [7].

In the second group solvents (ethanol, methanol, water and DMSO) of the thermodynamic hydrophilicity value being lower than 2.3 kJ/mol the hydrogen bond contribution is rather large allowing the dry protein to swell even at room temperature. At close thermodynamic hydrophilicity in proton accepting media the potential barrier of swelling is higher comparative to proton donating ones. Markedly slow swelling of albumin in DMSO in comparison with that in water and total lack of swelling in pyridine in contrast to ethanol exemplify this proposition.

Thus it permits to conclude that the solvent thermodynamic hydrophilicity and proton donating ability would be principal factors controlling the stability of dry solid chymotrypsin and kinetics of its swelling in anhydrous organic liquids and water at room temperature.

Acknowledgement

The authors gratefully acknowledge the Russian Foundation for Basic Researches (grant 98-03-32102) and the

Centre for Fundamental Natural Sciences at St.-Petersburg State University (grant 97-0-9.3-283) for financial support.

References

1. Klibanov, A.M. (1989) *Trends in Biochem. Sci.*, **14**, 141–144.
2. Zaks, A. and Klibanov, A.M. (1988) *J. Biol. Chem.*, **263**, 8017–8021.
3. Battistel, E. and Bianchi, D. (1994) *J. Phys. Chem.*, **98**, 5368–5375.
4. Dabulis, K. and Klibanov, A.M. (1992) *Biotechnol. Bioeng.*, **39**, 176–185.
5. Almarsson, O. and Klibanov, A.M. (1996) *Biotechnol. Bioeng.*, **49**, 87–92.
6. Fitzpatrick, P.A. and Klibanov, A.M. (1991) *J. Am. Chem. Soc.*, **113**, 3166–3171.
7. Borisover, M.D., Zakharychev, D.V., and Solomonov, B.N. (1999) *J. Therm. Analysis and Calorimetry*, **55**, 85–92.
8. Zakharychev, D.V., Borisover, M.D., and Solomonov, B.N., (1995) *Russ. J. Phys. Chem. in English Translation*, **69**, 162–166.
9. Borisover, M.D., Sirotkin, V.A., and Solomonov, B.N. (1996) *Thermochim. Acta*, **284**, 263–277.
10. Sirotkin V.A., Borisover, M.D., and Solomonov, B.N., (1997) *Biophys. Chem.*, **69**, 239–248.
11. Griebenow, K. and Klibanov, A.M. (1997) *Biotechnol. Bioeng.*, **53**, 351–362.
12. Dong, A., Meyer, J.D., Kendrick, B.S., Manning, M.C., and Carpenter, J.F., (1996) *Arch. Biochem. Biophys.*, **334**, 406–414.
13. D.D. Perrin, W.L.F. Armarego, and D.R. Perrin (1980) *Purification of Laboratory Chemicals.* Oxford: Pergamon Press.
14. *Handbook of Chemistry and Physics. 58th edition*, (1977–1978) (Weast, R.C., ed.). CRC Press Inc., Cleveland, Ohio.
15. Dong, A., Huang, P., and Caughey, W.S., (1990) *Biochemistry*, **29**, 3303–3308.
16. Gribenow, K. and Klibanov, A.M. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 10969–10976.
17. Constatntino, H.R., Griebenow, K., Mishra, P., Langer, R., and Klibanov, A.M. (1995) *Biochim. Biophys Acta*, **1253**, 69–74.
18. Laitinen, H.A. and Harris, W.E. (1975) *Chemical analysis,* 2nd edition. McGraw-Hill, New York.
19. Sirotkin, V.A., Zinatullin, A.N., Solomonov, B.N., Faizullin, D.A., and Fedotov, V.D. (2000) *Russ. J. Phys. Chem. in English translation*, **74**, 743–748.
20. Raichardt, C. (1988) *Solvent and solvent effects in organic chemistry*, 2nd ed. VCH, Weinheim.
21. Laane, C., Boeren, S., Vos, K., and Veeger, C. (1987) *Biotechnol. Bioeng.*, **30**, 81–87.
22. Gregory, R.B. (1995) *in Protein-solvent interaction.* (R.B. Gregory, ed). Dekker, New York. pp. 191–264.
23. Borisover, M.D., Sirotkin, V.A., and Solomonov, B.N. (1995) *Thermochim. Acta,* **256**, 175–183.
24. Prausnitz, J.M. (1969) *Molecular thermodynamics of fluid-phase equilibria*. Prentice-Hall, Inc.