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Interaction enthalpies of solid bovine pancreatic α-chymotrypsin with organic solvents: comparison with FTIR-spectroscopic data

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Abstract

Calorimetric heat effects and integral absorbance changes observed in the FTIR spectra were measured at immersing solid bovine pancreatic α-chymotrypsin in organic solvents and water at 298 K. Enthalpy changes upon the immersion of the enzyme in different media are in a good linear correlation with the corresponding IR-absorbance changes. Based on calorimetric and FTIR data, all the solvents were divided into two groups. The first group of solvents includes carbon tetrachloride, benzene, nitromethane, acetonitrile, 1,4-dioxane, *n*-butanol, *n*-propanol and pyridine in which no significant heat evolution and structural changes were found at the solid enzyme immersion. Second group of the solvents includes dimethyl sulfoxide, methanol, ethanol, and water. Immersion into these media, results in the solid protein swelling and involves significant exothermic heat evolution and structural changes in the protein. Dividing of different media in these two groups is in a qualitative correlation with the solvent hydrophilicity which is defined as partial excess molar Gibbs free energy of water at infinite dilution in a given solvent. The first group of solvents includes liquids with hydrophilicity exceeding 2.7 kJ/mol. The hydrophilicity of the second group solvents is <2.3 kJ/mol. Hydrogen bond donating ability of the solvents assists in the protein swelling. Hydrogen bonding between protein and solvent is assumed to be a main factor controlling the swelling of solid protein preparation in the solvents at room temperature.

Keywords: Bovine pancreatic α-chymotrypsin; Organic solvent; Interactions enthalpies; Isothermal immersion calorimetry; FTIR-spectroscopy

1. Introduction

It has been firmly established that solid proteins immersed in organic solvents with low water contents demonstrate some remarkable properties: catalysis of the reactions not feasible in aqueous media [1,2], greatly enhanced thermostability [3,4] and molecular bioimprinting [5,6]. This powerful biotechnological potential of proteins in organic media depends strongly on the nature of organic solvent. However, ''there is still inadequate knowledge about protein–solvent interactions. This is definitely a rate-limiting step in the further growth of this area'' [7].

To understand the effects of organic media on properties and catalytic activity of proteins, we should elucidate the state of protein macromolecules subjected to such extraordinary conditions. This aim may be achieved by studying the relationships between the thermodynamic and structural parameters of formation

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of the solid protein–organic solvent systems. Due to the ability to monitor the thermodynamic and structural changes in such heterogeneous systems, the combination of the calorimetry and IR-spectroscopy has a great potential in examining factors governing the solid protein–solvent interactions.

Earlier, we proposed a calorimetric approach for examining the processes that occur on immersing the solid proteins in water–organic mixtures [8–10]. This approach involved the measurement of the enthalpies corresponding to the formation of the ''protein+ liquid'' heterogeneous systems. It was suggested [10,11] that the enthalpy of formation of HSA suspensions in water–organic mixtures is controlled mainly by two processes. The first is the water desorption/ sorption which may follow the Langmuir kind trend. Thermodynamics of the water sorption by the protein in organic solvents was evaluated using the Langmuir model. Solvent effect on thermodynamic parameters of the water sorption by the protein was found in a strong correlation with the water solvation thermodynamics in organic media [10]. The second process attributed to the protein swelling in water–organic mixture [10,11] is associated with the significant heat effects and increase in the surface area accessible for sorption of water. However, the role of protein conformational changes in calorimetric events observed on suspending the protein in organic media was not justified.

FTIR spectroscopy has been successfully applied for examination of the secondary structure of proteins in organic solvents [12,13]. It was shown that some organic solvents induce conformational changes in the suspended protein; in some cases, the introduction of lyophilized proteins into anhydrous organic media has no effect on the secondary structure. However, factors governing the protein structure in low-water–organic media are not really clear.

Therefore, in the present study, we want to combine isothermal immersion calorimetry and IR-spectroscopy in the examination of the protein–solvent interactions. The objective of this combined calorimetric and IR study is to elucidate the relations between the effect of solvent nature on protein secondary structure and heat effects of interaction of solid protein with organic liquids.

As is known, water sorbed by proteins in organic solvents may significantly influence on the catalytic properties [14], protein conformation [13] and thermostability [3,4], and enthalpies of suspension formation [8–10]. Hence, in order to focus on the protein–organic solvent interactions, essentially dry protein (dehydrated at water activity <0.01) immersed in highly dehydrated pure organic solvents was investigated by calorimetry and IR-spectroscopy. Behavior of solid protein in organic solvents was also compared with that in water environment.

2. Experimental

2.1. Materials

Bovine pancreatic α-chymotrypsin (Sigma, No. C 4129, essentially salt free; EC 3.4.21.1; specific activity of 52 units/mg of solid) was used without further purification. Organic solvents (reagent grade, purity > 99%) were purified and dried according to the recommendations [15] and than were stored over dry 3 Å molecular sieves for at least 24 h prior to use. Water used was doubly distilled.

2.2. 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 by flushing the air dried over P_2O_5 powder. Relative vapor pressure over P_2O_5 at 298 K does not exceed 0.01 [16]. The protein film was flushed until no further spectral changes were detected in the 3450 cm^{-1} water absorbance region and amide I contour in this side represented a smooth line without any visible shoulders. The cell was then filled by the chosen organic solvent. Spectra were recorded as a function of time until equilibrium was achieved. Then, spectra of the solvent without protein sample were recorded and subtracted from the protein + solvent spectra.

Protein spectra in water environment have been obtained on protein films by flushing damp air at 99% relative humidity. Spectrum of liquid water was then subtracted from the spectra of wet films in

accord with the criteria described in [17]. Changes of the secondary protein structure have been analyzed using the established correlation between secondary structure elements in proteins and peak positions in the amide I spectra [18,19].

2.3. Calorimetric measurements

Commercial lyophilized protein preparations were dried under vacuum using microthermoanalyzer (Setaram, MGDTD-17S) at 298 K and 0.1 Pa until constant sample weight. Water content of the dried protein was estimated as 0.003 ± 0.003 g of water per gram of protein by the Karl Fischer titration method [20]. Calorimetric heat effects on immersing the solid protein powder into organic solvents and water were measured at 298 K with a Setaram BT-215 calorimeter according to the described procedure [8]. 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. Calorimeter was calibrated using the Joule effect and tested with dissolving sodium chloride in water according to the recommendations [21]. The enthalpy changes on dissolution of the α-chymotrypsin (CT) powders in water were measured at protein concentration of 1 g/l.

2.4. Solubility control

Solubility of solid α-chymotrypsin was controlled by optical density measurements of supernatants on Specord M-40 spectrophotometer at 260–300 nm (sensitivity limit of assay −0.01 mg/ml). No protein was observed in liquid phase. 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 dehydrated organic solvents.

In our experiments, DMSO, while not dissolving the proteins, has transformed the samples to transparent dense gel. It is known that some non-aqueous solvents, for example, DMSO and formamide, can dissolve proteins [22,23]. However, the mechanism of protein dissolving potential of anhydrous organic solvents is still not clear [23]. Protein solubility in organic solvents may depend strongly on variety of factors such as pH, temperature, solvent humidity, electrolyte additives [22–25]. Probably, deep dehydration of the proteins and absence of salts may strongly decrease solubility of macromolecules in anhydrous organic solvents.

2.5. 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. $\overline{G}^{E_\infty}$ is determined by Eq. (1) [26]:

$$\overline{G}^{E_\infty} = RT \ln(\gamma_w^\infty) \tag{1}$$

where γ_w^∞ is a mole fraction basis activity coefficient for water at infinite dilution. Reference state for the activity coefficient is pure liquid water ($\gamma_w \to 1$ at $x_w \to 1$). $\overline{G}^{E_\infty}$ becomes more negative in more hydrophilic solvents. The $\overline{G}^{E_\infty}$ values are presented in Table 1.

The water activity coefficients were calculated by one of the following two methods:

1. It is expected that water activity coefficients in the water immiscible hydrophobic solvents do not depend on the water concentration over all the solubility range [27]. Hence, γ_w^∞ may be estimated using Eq. (2):

$$\gamma_w^\infty = \frac{1}{x_w} \tag{2}$$

   where x_w is the mole fraction solubility of water in the hydrophobic solvent. This method was used for estimating the water activity coefficients in carbon tetrachloride and benzene. Water solubilities in carbon tetrachloride at 297 K and benzene at 299 K were taken from [28]. The temperature effect on the water solubility in carbon tetrachloride and benzene was tested using the UNIFAC model [29]. The temperature difference of 1 K involves no more than 5% change of the activity coefficient value.
2. Water activity coefficients in hydrophilic solvents were calculated using literature data on the vapor–liquid equilibrium according to the Eq. (3):

$$\gamma_w = \frac{y_w P_t}{x_w P_o} \tag{3}$$

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

Table 1
Enthalpy changes (ΔH_{tot}) on immersing solid α-chymotrypsin into organic solvents and water, normalized areas (ΔA) corresponding to the positive parts of solvent-induced difference spectra and the partial molar excess Gibbs free energies ($\overline{G}^{E_\infty}$) of water at infinite dilution 298 K[a]

Number	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	Pyridine	−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

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

Vapor–liquid equilibrium data for water mixtures with pyridine, 1,4-dioxane, methanol, ethanol, *n*-butanol, *n*-propanol, and acetonitrile are from [30]. Vapor–liquid equilibrium data for water mixtures with nitromethane and DMSO are from [31,32]. Water activity coefficients at infinite dilution γ_w^∞ were estimated by extrapolation of activity coefficients γ_w at high water concentrations by a polynomial expression.

2.6. Water contents of organic solvents

Humilities of solvents, determined by the Karl Fischer titration [20], were 0.02 mol/l in 1,4-dioxane, 0.01 mol/l in *n*-butanol, 0.02 mol/l in *n*-propanol, 0.005 mol/l in nitromethane, 0.022 mol/l in acetonitrile, 0.021 mol/l in ethanol, 0.07 mol/l in pyridine, 0.23 mol/l in DMSO, 0.1 mol/l in methanol. Water activities a_w calculated as: $a_w = \gamma_w x_w$, did not exceed 0.01. Water contents of carbon tetrachloride and benzene were <0.001 mol/l.

3. Results and discussion

3.1. Enthalpy and integral absorbance changes on the immersion of solid α-chymotrypsin in organic solvents

Typical heat evolution curves recorded on immersing the solid enzyme preparation into DMSO and water are given in Fig. 1. As an example, the area corresponding to the total heat effect (ΔH_{tot}) evolved at dissolution of solid enzyme in water is shaded.

Fig. 2A shows CT spectra of carbon tetrachloride, benzene, acetonitrile, 1,4-dioxane, *n*-butanol, *n*-propanol and pyridine. Only subtle amide I absorbance changes (if any) relative to protein spectrum in dry air may be seen in this case. Fig. 2B demonstrates much more considerable alterations observed on the immersion of protein in methanol, ethanol, DMSO and water.

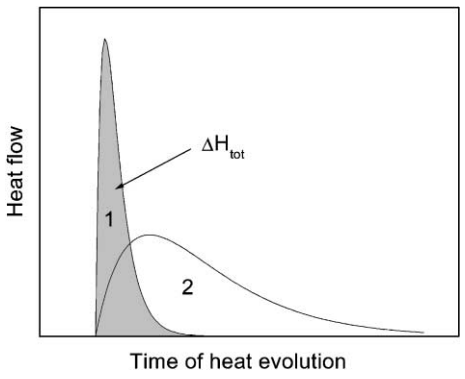

Fig. 1. Calorimetric curves recorded on contacting solid α-chymotrypsin with water (1) or DMSO (2). As an example, area corresponding to the total heat effect (ΔH_{tot}) of aqueous dissolution is shown as shaded.

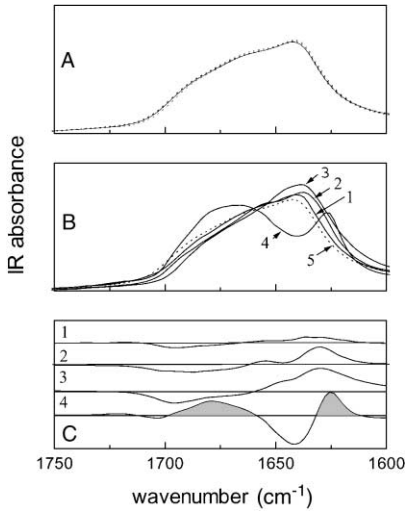

Fig. 2. Amide I spectra of α-chymotrypsin films — (A) in dry air (solid line), anhydrous organic solvents (dashed lines; including carbon tetrachloride, benzene, acetonitrile, 1,4-dioxane, *n*-butanol, *n*-propanol and pyridine); (B) in ethanol: (1) methanol (2), water (3), DMSO (4), dry air (5); (C) difference spectra; numbering corresponds to B; curves 1–4 are shifted for clarity. As an example, the area (ΔA) of difference spectrum in DMSO is shown as shaded.

The overall changes in the secondary structure of protein in solvents as compared with the dry protein state were quantitatively characterized by the positive area of solvent-induced spectral difference in the amide I region (ΔA) of protein. For example, such spectral changes induced by DMSO are shown as shaded in Fig. 2C. All ΔA values were normalized on the amide I peak absorbance of the protein sample recorded in the dry state.

The ΔA values, enthalpies of the immersion of solid enzyme in organic solvents and enthalpy changes on dissolution of the CT powder in water (ΔH_{tot}) are presented in Table 1.

Interaction of a protein with its environment depends on a variety of factors. It is evident that measured calorimetric heats and spectral alterations have to be quite complex quantities. However, a good linear correlation between the ΔH_{tot} values and spectral ΔA parameters was found (Eq. (4)). This linear correlation is shown in Fig. 3:

$$-\Delta H_{tot} = (-8.9 \pm 6.3) + (12.2 \pm 1.3)\Delta A \quad (4)$$

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

Intercept in Eq. (4) is small and close to 0. This shows that calorimetric heat effects reflect mainly the structural rearrangements in the protein. On the other hand, the good linear relationship between ΔH_{tot} and ΔA shows that irrespective on the solvent nature and character of structural changes, the exothermicity of the solid protein–solvent interaction increases with the degree of rearrangement of

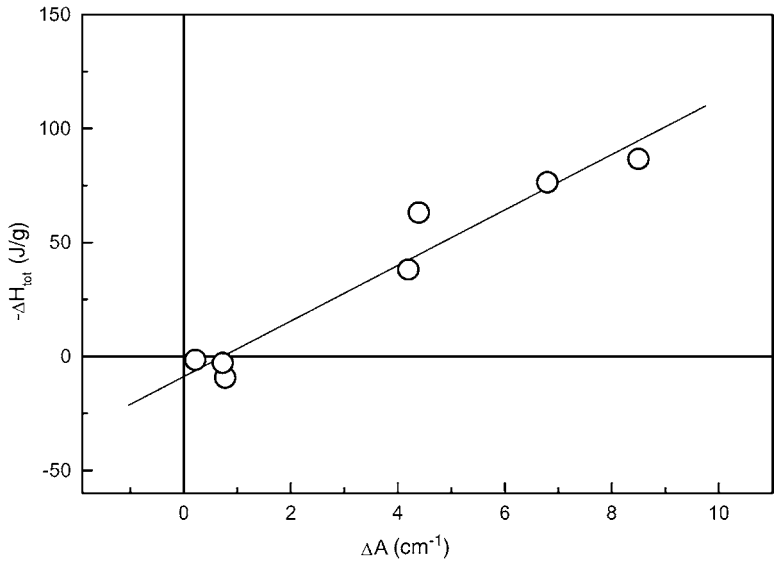

Fig. 3. ΔH_{tot} values plotted against integral absorbance changes ΔA. The data correspond to Table 1.

the structure of the dehydrated protein (as measured by ΔA).

3.2. Influence of solvent nature on enthalpy and integral absorbance changes

Correlation between the solvent effect on the ΔH_{tot} and ΔA values and solvent properties was examined by using the solvent dielectric constant [33], solvent hydrophobicity expressed as $\log P$ (where P is the partition *n*-octanol–water coefficient) [34], solvent molar volume, solvent donor and acceptor numbers [33], and solvent hydrophilicity based on the partial molar excess Gibbs energy $\overline{G}^{E_\infty}$ of water in a given solvent at infinite dilution (Table 1).

No correlation was found for chymotrypsin when ΔH (ΔA) was plotted against any one solvent parameter such as dielectric constant, hydrophobicity, molar volume, donor and acceptor numbers (Fig. 4A–E). Significant discrepancies exceeding the experimental errors may be seen in Fig. 4A–E.

Reasonable trend may be obtained when ΔH (ΔA) was plotted against partial excess molar Gibbs free energy (Fig. 5). As is seen from Fig. 5, solvents may be divided in two groups according to the measured calorimetric heats and observed integral absorbance changes. This separation agrees well with the solvent hydrophilicity.

The first group of solvents includes liquids with $\overline{G}^{E_\infty} > 2.7$ kJ/mol, i.e. carbon tetrachloride, benzene, nitromethane, acetonitrile, 1,4-dioxane, *n*-butanol, *n*-propanol and pyridine. The heat evolved upon the interaction of solid protein with the first group solvents is close to 0. The second group consists of the most hydrophilic liquids such as water, DMSO, methanol and ethanol. Their $\overline{G}^{E_\infty}$ values are lower than 2.3 kJ/mol. Immersion of solid protein into the second group solvents is followed by significant exothermic enthalpy changes. The most exothermic heat effects are observed in water and DMSO.

Spectral changes ΔA observed in different media fit to the same solvent classification. Solvents of the first group do not induce the significant changes in the protein secondary structure as compared with the initial state in dried solid protein, thus producing $\Delta A \approx 0$. Immersion of the solid protein into the second group solvents is followed by significant changes in the secondary protein structure ($\Delta A > 4.0\ \mathrm{cm}^{-1}$). The most significant structural changes are observed in water and in DMSO.

Interaction of the solid CT with solvents of the second group can be hardly considered as a simple physical adsorption on surface of solid protein phase. Such a physical adsorption would not induce significant structural changes in the sorbent. Significant changes in the protein secondary structure and exothermic heat effects observed for proteins immersed in the second group solvents are indicative for the solid biopolymer swelling in organic low molecular liquids (i.e. dissolution of the organic molecules in solid protein phase). Aqueous swelling results finally in the protein dissolution.

Solid protein does not demonstrate such a swelling in the solvents of the first group. The possible reason may be that dehydrated solid protein is in the kinetically ''frozen'' state [35]. The potential barrier for swelling of such dried protein may be quite high, thus preventing swelling in this group of solvents. This hypothesis is supported by results of [36] in which thermostability of HSA suspended in *n*-hexane–pyridine mixtures has been examined by DSC. So, it was found that heating of the HSA suspension in pyridine (and pyridine–*n*-hexane mixtures) results in the exothermic peak. Such a peak is indicative that HSA immersed in pyridine, which is the first group solvent, is in the non-equilibrium state at 298 K.

3.3. Influence of hydrogen bond donating ability

Hydrogen bond donating ability of a solvent molecule seems to be the important factor lowering potential barrier of swelling of solid protein. For example, hydrophilicities of EtOH and pyridine are close to 2.3 and 2.7 kJ/mol, respectively. The ΔA and ΔH_{tot} values are nearly 0 in the hydrogen bond accepting pyridine. However, immersing solid chymotrypsin into the hydrogen bond donating ethanol is followed by the significant structural change and exothermic heat effect (Table 1).

Qualitative confirmation of the role of the hydrogen bond donating ability of solvents in the protein swelling may be also seen in comparing the kinetics of heat evolution on the protein immersion. For example, water and DMSO demonstrate lowest $\overline{G}^{E_\infty}$ values that are 0 and -3 kJ/mol, respectively. Heat evolution on

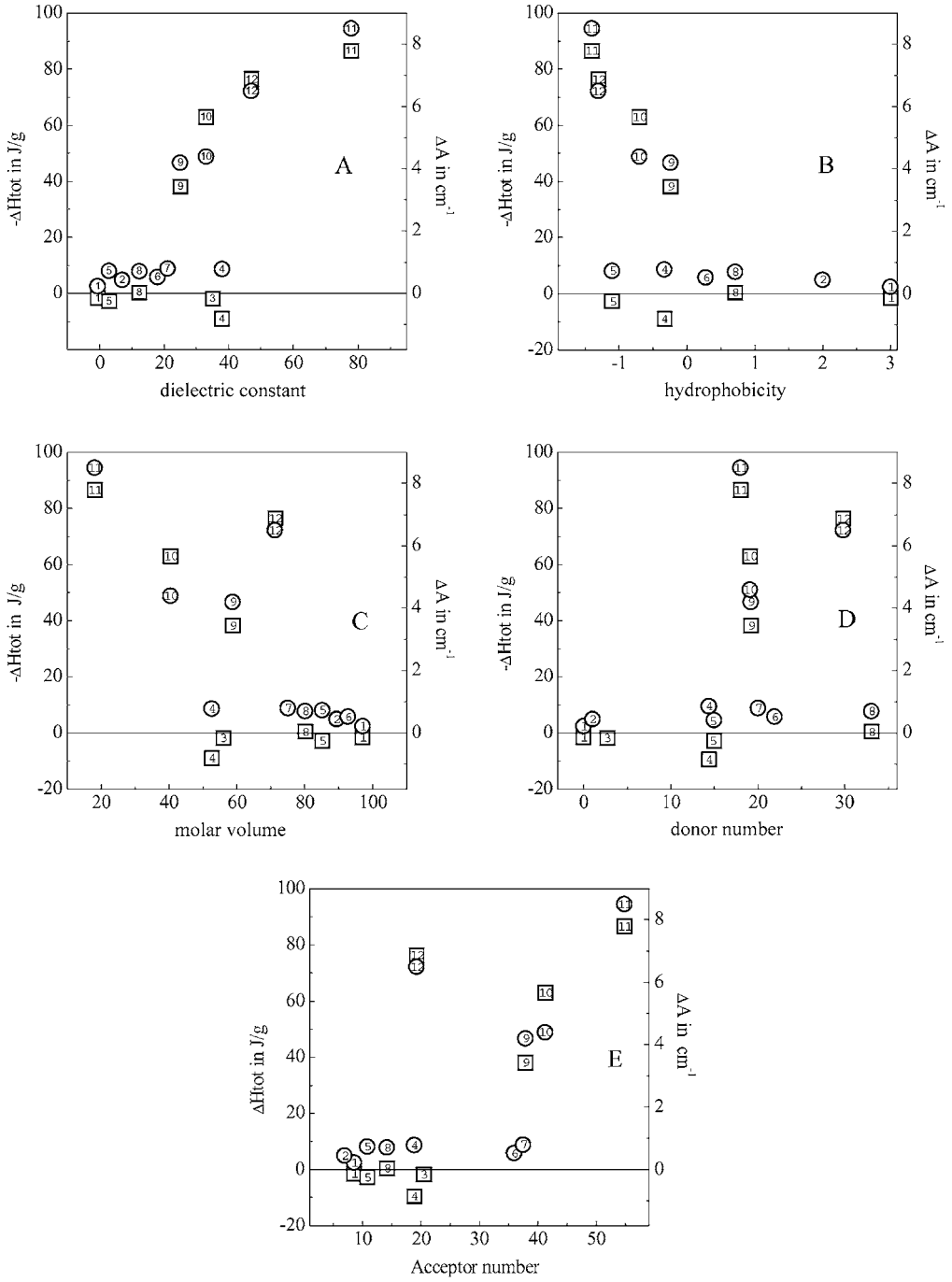

Fig. 4. Enthalpy changes ΔH (squares) and spectral differences ΔA (circles) plotted against solvent's dielectric constant (A), hydrophobicity (B), molar volume (C), donor (D) and acceptor number (E).

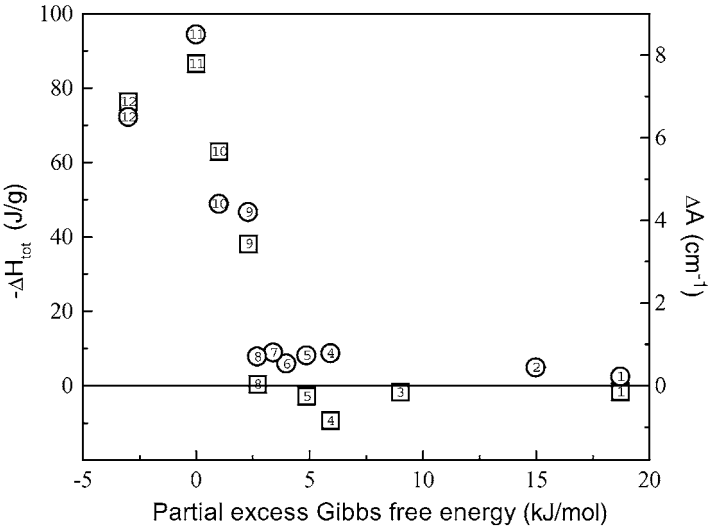

Fig. 5. Enthalpy changes ΔH (squares) and spectral differences ΔA (circles) plotted against partial excess molar Gibbs free energy of water at infinite dilution in a series of solvents at 298 K.

the CT dissolution in water was completed within 40 min. Heat evolution at the interaction between CT and DMSO is completed within 2.5 h. Slow heat evolution on immersing the partially hydrated (10% w/w) HSA into water–DMSO mixtures was observed also in [37]. It was found that increase of the water contents in DMSO reduced significantly the time of interaction between HSA and DMSO.

3.4. Structural events on swelling in the second group solvents

DMSO was the only hydrogen bond accepting solvent resulting in the structural changes in dehydrated solid α-chymotrypsin. However, the kinetics of structural rearrangements in DMSO was much slower than in less hydrophilic water, methanol and ethanol. This supports hypothesis that hydrogen bond donating ability of solvents contributes significantly in decreasing the potential barrier of the solid protein swelling.

Hydrogen bond donating and accepting solvents affect differently on the protein structure. Contact between CT and alcohols results not only in generating some helical structure but also in the subsequent formation of more extensive β-sheets (Fig. 2B and 2C). Swelling of CT in DMSO shows, as initial step, relatively fast randomizing of the protein structure followed by slow conversion to extensive β-aggregation.

3.5. Classification of solvents on their solid α-chymotrypsin swelling ability

Behavior of solid enzyme in organic solvents is conceptualized using the solvent hydrophilicity and hydrogen bond donating ability as in Fig. 6. It depicts the potential energy changes along the reaction coordinate for three typical cases considered in the work. Hydrogen bonding between a protein and a solvent results in the decrease of the potential energy barrier of the solid protein swelling. According to this conception, the total energy of formation of the ''solid protein + organic solvent'' system is in correlation with the decrease of the potential barrier for the protein swelling.

From the other hand, it is known that $\overline{G}^{E_\infty}$ measures the deviation of water state in organic solvents from ideality [26]. This deviation mainly arises from the formation of hydrogen bonds between water and organic molecules. As such, the $\overline{G}^{E_\infty}$ values may be considered as a measure of intensity of hydrogen bonding between water and solvent molecules. Strongly negative $\overline{G}^{E_\infty}$ value, for example, for DMSO

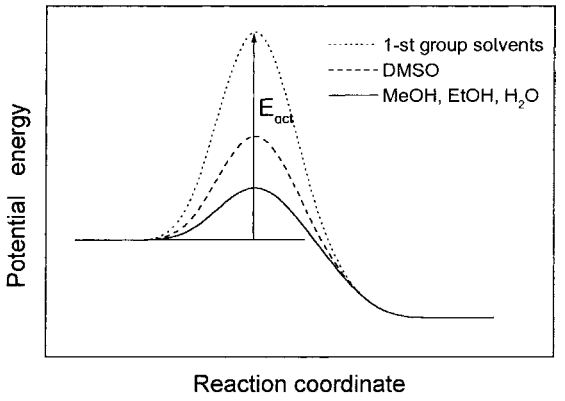

Fig. 6. Qualitative scheme describing the dependence of potential barrier of solid α-chymotrypsin swelling on the nature of solvent.

indicates strong hydrogen bonding between water and solvents. And vice versa strongly positive $\overline{G}^{E_\infty}$ value for benzene indicates that hydrogen bonding contribution is negligible in this system. It is hypothesized that contribution from hydrogen bonding between protein and organic solvent to the total measured energy (immersion heat) correlates with the $\overline{G}^{E_\infty}$ values.

According to this criterion, solvents with the hydrophilicity >2.7 kJ/mol are not effective in hydrogen bonding with a protein. Hence, the potential barrier of the protein swelling is high in these solvents. This status is diagrammed by upper curve in Fig. 6. Raise of the temperature may help in passing this potential barrier as it was suggested for explaining the exothermic DSC peaks for HSA suspended in pyridine [36].

Second group solvents (i.e. ethanol, methanol, water) demonstrate high hydrophilicity ($\overline{G}^{E_\infty} < 2.3$ kJ/mol) and effective in the hydrogen bond formation both with water and the protein, thus resulting in a swelling of protein at room temperature. Bottom curve in Fig. 6 corresponds to this case.

Hydrogen bond donating ability of solvents strengthens their swelling efficacy towards dehydrated protein. Therefore, at close solvent hydrophilicity in hydrogen bond donating media, the potential barrier of swelling is lower comparative to hydrogen bond accepting ones. This was exemplified by slow swelling of α-chymotrypsin in DMSO as compared with that in water and by the absence of swelling in pyridine as compared with ethanol. Effect of hydrophilic hydrogen bond accepting solvents such as DMSO is shown in Fig. 6 by the middle curve.

4. Conclusion

We conclude that the combination of isothermal immersion calorimetry and FTIR-spectroscopy provides an effective experimental approach for estimating interactions that occur on immersing solid protein into organic solvents. The results obtained demonstrate that solvent potential to form the hydrogen bonding appears an important factor controlling the swelling stability of solid α-chymotrypsin in the solvents under study.

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