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Effect of dioxane on the structure and hydration–dehydration of α-chymotrypsin as measured by FTIR spectroscopy

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Abstract

A new experimental approach based on FTIR spectroscopic measurements was proposed to study simultaneously the adsorption/desorption of water and organic solvent on solid enzyme and corresponding changes in the enzyme secondary structure in the water activity range from 0 to 1.0 at 25 °C. The effect of dioxane on the hydration/dehydration and structure of bovine pancreatic α-chymotrypsin (CT) was characterized by means of this approach. Dioxane sorption exhibits pronounced hysteresis. No sorbed dioxane was observed at low water activities (a_w<0.5) during hydration. At a_w about 0.5, a sharp increase in the amount of sorbed dioxane was observed. Dioxane sorption isotherm obtained during dehydration resembles a smooth curve. In this case, CT binds about 150 mol dioxane/mol enzyme at the lowest water activities. Three different effects of dioxane on the water binding by the initially dried CT were observed. At a_w<0.5, water adsorption is similar in the presence and absence of dioxane. It was concluded that the presence of dioxane has little effect on the interaction between enzyme and tightly bound water at low a_w. At a_w>0.5, dioxane increases the amount of water bound by CT during hydration. This behavior was interpreted as a dioxane-assisted effect on water binding. Upon dehydration at low water activities, dioxane decreases the water content at a given a_w. This behavior suggests that the suppression in the uptake of water during dehydration may be due to a competition for water-binding sites on chymotrypsin by dioxane. Changes in the secondary structure of CT were determined from infrared spectra by analyzing the structure of amide I band. Dioxane induced a strong band at 1628 cm^{-1} that was assigned to the intermolecular β-sheet aggregation. Changes in the intensity of the 1628 cm^{-1} band agree well with changes in the dioxane sorption by CT. An explanation of the dioxane effect on the CT hydration and structure was provided on the basis of hypothesis on water-assisted disruption of polar contacts in the solid enzyme. The reported results demonstrate that the hydration and structure of α-chymotrypsin depend markedly on how enzyme has been hydrated—whether in the presence or in the absence of organic solvent. A qualitative model was proposed to classify the effect of hydration history on the enzyme activity-a_w profiles.

Keywords: Bovine pancreatic α-chymotrypsin; Thin film; Enzyme hydration; Secondary structure; Organic solvent sorption; Hysteresis phenomenon; Fourier transform infrared spectroscopy

1. Introduction

The hydration of proteins (enzymes) is a phenomenon of considerable fundamental importance and practical interest. It is well known that the interaction of water with proteins plays a key role in determining the structure and functions of proteins [1–3]. Knowledge of processes occurring upon the hydration or dehydration of proteins is also very important in biotechnological and pharmaceutical applications of proteins such as their use as biocatalysts [4–8], biosensors [9,10] and selective adsorbents [11,12] in low water organic solvents. There are many advantages in employing organic solvents for enzymatic processes, including high solubility of organic substrates, synthesis of useful chemicals (for example, chiral drug molecules, emulsifiers, modified fats and oils, flavor esters), suppression of undesirable side reactions caused by water and increased thermostability. However, in general, the enzyme activity in organic solvents is a complex function of the

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water content in the organic media [13,14] and the hydration "history" of an enzyme [15,16]. Hence, there is considerable interest in understanding the intermolecular interactions and conformational rearrangements that occur upon the hydration–dehydration of solid enzymes in the presence of organic solvents.

The hydration of solid proteins in organic solvents was investigated in many studies [17–21]. It was concluded that when the water sorption is plotted as a function of thermodynamic water activity (a_w), the sorption isotherms obtained in organic solvents are similar to those recorded in the absence of organic solvents at low a_w values [22]. At higher water activities ($a_w > 0.4$), the organic solvents caused deviations. Hydrophobic solvents (for example, benzene [22] and ethyl acetate [23]) usually increased the amount of water bound to the protein (at fixed a_w) while hydrophilic solvents (for example, ethanol [22] and propanol-1 [20]) had the opposite effect. The different mechanisms of hydration of human serum albumin (HSA) in various organic liquids were observed from the combined calorimetric and water sorption measurements [24–27]. It was shown that depending on the organic solvent and its water content, the immersion of solid protein into the water–organic mixtures may involve both the water sorption on the solid protein and the second exothermic process. This second process was considered to include the rupture of the protein–protein contacts in the solid phase induced by protein–organic solvent or/and protein–water interactions. However, there are no reports on the hydration–dehydration cycle for proteins obtained in the presence of organic solvent.

Infrared spectroscopy is one of the effective methods for analyzing the structure of proteins in various environments, including aqueous and non-aqueous media. This method has been successfully used in studying the secondary structure of proteins in various states, including solid preparations with various degrees of humidity [28–34] and solid proteins immersed into pure organic solvents and water–organic mixtures [35,36].

Infrared spectroscopy is also effective in studying the hydration of proteins [1,2,33]. The relationship between hydration and acetonitrile vapor sorption by human serum albumin was studied by FTIR spectroscopy [37]. By means of the combined calorimetric and FTIR spectroscopic measurements the structure and stability of dehydrated chymotrypsin and HSA were recently examined in a series of anhydrous organic liquids, including hydrocarbons, alcohols and hydrogen bond accepting solvents [38,39]. It was shown that solvent potential to form hydrogen bonds appears to be an important factor controlling the stability of dehydrated proteins in organic media. However, no attempt has been made to study simultaneously both the hydration–dehydration of solid enzyme in the presence of organic solvent and the corresponding structural changes over the whole range of water activity.

In the present work a new experimental approach based on FTIR spectroscopic measurements was proposed to study simultaneously the adsorption/desorption of water and organic solvent on solid enzyme and corresponding changes in the secondary structure over the whole range of water activity at 25 °C. By means of this approach, the adsorption and desorption of pure water and water–dioxane vapor mixtures on bovine pancreatic α-chymotrypsin were investigated. Sorption data were compared with the structural changes that occur on the interaction of solid enzyme with water and organic molecules. The aim of this combined study is to elucidate the mechanism of molecular processes that occur upon the hydration and dehydration of solid enzyme in the presence of organic solvent.

Dioxane was selected as a probe organic compound because it is capable of forming strong hydrogen bonds with various hydrogen donors. However, in contrast to water, it has no evident hydrogen bond donating ability. Bovine pancreatic α-chymotrypsin was used as a model enzyme because it is one of the most researched enzymes in aqueous [40,41] and nonaqueous [4–8] enzymology.

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. Dioxane (reagent grade, purity >99%) was purified and dried according to the recommendations [42] and was stored over molecular sieves (3 Å) for at least 24 h prior to use. Water used was doubly distilled.

2.2. Thermodynamic activities of water and dioxane

Water activity (a_w) in organic solvent was calculated using the Eq. (1):

$$a_w = \gamma_w x_w, \tag{1}$$

where x_w is the mole fraction of water in the solution; and γ_w is the activity coefficient of water (in mole fractions; the standard state is pure water). Water content in dioxane (x_w) was measured using Karl Fisher method according to the recommendations [27,43].

Water activity coefficients in organic solvent γ_w were calculated from literature data on the vapor–liquid equilibrium [44] by Eq. (2):

$$\gamma_w = \frac{y_w P_{tot}}{x_w P_w^o}, \tag{2}$$

where y_w is the mole fraction of water in vapor phase, P_{tot} is the total pressure, P_w^o is the saturated vapor pressure of pure water at the same temperature and x_w is the mole fraction of water in the liquid phase.

Dioxane activity (a_{dio}) was calculated using the Eq. (3):

$$a_{dio} = \gamma_{dio} x_{dio}, \quad (3)$$

where x_{dio} is the mole fraction of dioxane in the solution; and γ_{dio} is the activity coefficient of water (in mole fractions; the standard state is pure dioxane).

Dioxane activity coefficients in organic solvents (γ_{dio}) were calculated by Eq. (4):

$$\gamma_{dio} = \frac{y_{dio} P_{tot}}{x_{dio} P^{o}_{dio}}, \quad (4)$$

where y_{dio} is the mole fraction of dioxane in vapor phase; P_{tot} is the total pressure; P^{o}_{dio} is the saturated vapor pressure of pure dioxane at the same temperature and x_{dio} is the mole fraction of dioxane in the liquid phase.

The thermodynamic activities of water and dioxane at 25 °C are presented in Fig. 1.

2.3. FTIR spectroscopic measurements

The infrared spectra were measured at 25 °C with a Vector 22 FTIR-spectrometer (Bruker) at 4 cm^{-1} resolution as described previously [37–39]. A schematic representation of the experimental set-up for FTIR spectroscopic measurements of the structural and sorption characteristics of solid enzyme is given in Scheme 1. The infrared spectra were obtained with glassy like protein films casted from 2% (w/v) water solution onto the CaF_2 window at room humidity and temperature. After mounting the window in the sample cell, the film was dehydrated by flushing air dried over P_2O_5 powder (Scheme 1, Route 1). Water activity over P_2O_5 at 25 °C does not exceed 0.01 [45]. The enzyme film was flushed until no further spectral changes were detected in the 3500 cm^{-1} water absorbance region and amide A contour on this side represented a smooth line without any visible shoulders. The spectrum of this sample was used as a reference spectrum for calculation of the difference spectra. The difference spectra were obtained according to the criteria described previously [29].

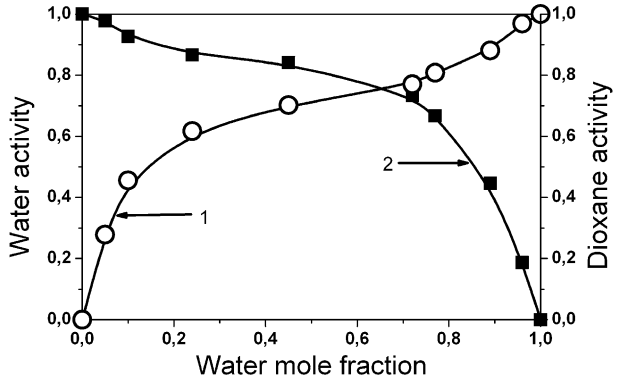
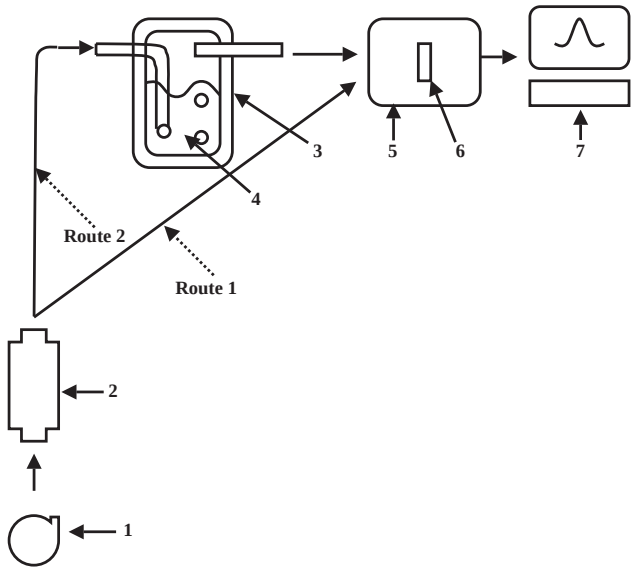


Fig. 1. Correlations between the thermodynamic activities of water and dioxane and water mole fraction in the liquid phase at 25 °C: (1) water activity; (2) dioxane activity.



Scheme 1. Schematic representation of the experimental set-up for FTIR spectroscopic measurements of structural and sorption characteristics of solid enzyme. The components of the experimental set-up: 1—air pump; 2—thermostated glass tube with drying agent; 3—thermostated saturator; 4—pure liquid water or water–organic mixture; 5—FTIR spectrometer; 6—thermostated sample cell; 7—computer.

Then, the sample was in situ exposed to pure water vapor or water–organic vapor mixtures. In the first case, pure water vapor consecutively flowed through the thermostated glass tube with a drying agent (P_2O_5), saturator filled with pure liquid water and then through the measuring cell containing the protein sample (Scheme 1, Route 2). The temperature of the sample cell was 25 °C. The water activity (a_w) in the vapor phase was adjusted by changing the difference between the temperature of the saturator and cell. Data on water vapor pressure at various temperatures were taken from [46].

In the second case, the air consecutively flowed through the thermostated glass tube with a drying agent (P_2O_5), saturator filled with a water–organic mixture and then through the sample cell (Scheme 1, Route 2). The temperature of the sample cell and saturator was 25 °C. The water activity (a_w) in the vapor phase was adjusted by changing the water activity in the liquid water–organic mixture.

Water sorption by chymotrypsin films was controlled in the region of OH stretching vibration band at 3500 cm^{-1}. Adsorbed dioxane has some intensive vibrational bands. The bands at 2966 and 2862 cm^{-1} were assigned to the CH_2 asymmetric and symmetric stretching vibrations, respectively. The band at 1455 cm^{-1} was assigned to the CH_2 bending vibrations. Dioxane sorption by solid enzyme was controlled at 1121 cm^{-1}, which is the most intensive band of adsorbed dioxane. This band was assigned to the stretching vibrations of the C–O group. Good linear correlations were observed between the relative absorbance values (D_x/D_o) at 2966, 2862 and 1455 cm^{-1} and the D_{1121}/D_o values (Fig. 2).

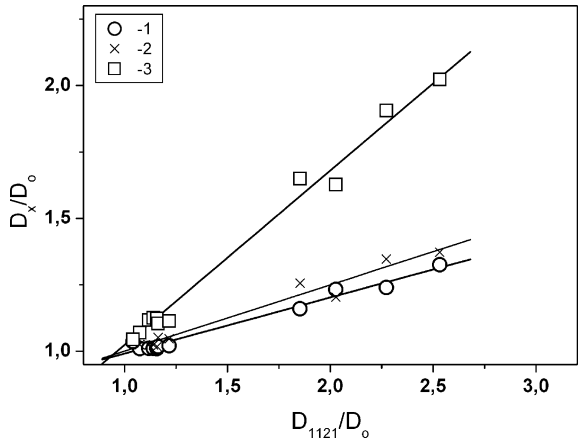


Fig. 2. Correlations between the relative absorbance values (D_x/D_o) at 1455, 2966 and 2862 cm^{-1} and the D_{1121}/D_o values: (1) 1455 cm^{-1}; (2) 2966 cm^{-1}; (3) 2862 cm^{-1}. D_o is the absorbance value for Film A (the initial state of spectroscopic measurements; Section 3.1).

$$D_{1455}/D_o = 0.78(0.02) + 0.21(0.01)*D_{1121}/D_o$$

Correlation coefficient $R=0.99$; number of experimental points $N=11$; standard error of estimation $s_o=0.02$.

$$D_{2966}/D_o = 0.75(0.03) + 0.25(0.02)*D_{1121}/D_o$$

Correlation coefficient $R=0.98$; number of experimental points $N=11$; standard error of estimation $s_o=0.03$.

$$D_{2862}/D_o = 0.37(0.05) + 0.66(0.03)*D_{1121}/D_o$$

Correlation coefficient $R=0.99$; number of experimental points $N=11$; standard error of estimation $s_o=0.05$.

Enzyme films were flushed by pure water vapor or water–organic vapor mixtures until no further changes were detected in the absorbance values at 3500 and 1121 cm^{-1}. Upon hydration and dehydration in the absence of dioxane no noticeable spectral changes were observed after 30 min. Upon hydration and dehydration in the presence of dioxane, the sorption equilibrium was reached after 1 h. This increase in the time required for the attainment of sorption equilibrium in the presence of dioxane is presumably due to pronounced conformational rearrangements and considerable changes in the mechanism of water and organic solvent sorption as described in Sections 3.1–3.5.

Supposing that during sorption the protein film swells predominantly due to increasing thickness, the enzyme hydration and sorption of dioxane vapor were calculated from the Eqs. (5) and (6):

$$h = 2.3S_{water}\varepsilon_{CT}/B_{water}D_{CT}, \quad (5)$$

where h is the chymotrypsin hydration, mol water mol^{-1} enzyme; S_{water} is the area of water absorbance band, cm^{-1}; ε_{CT} is the protein molar extinction at the maximum of Amide I band, l mol^{-1} enzyme cm^{-1}; B_{water} is the water integral molar extinction coefficient, l mol^{-1} water cm^{-2}; D_{CT} is the optical density at the maximum of amide I band. For pure water, it was taken that $B_{water}=96{,}000\pm1000$ l mol^{-1} water cm^{-2} [47].

$$A = 2.3S_{dioxane}\varepsilon_{CT}/B_{dioxane}D_{CT}, \quad (6)$$

where A is the sorption of dioxane, mol dioxane mol^{-1} enzyme; $S_{dioxane}$ is the area of organic solvent absorbance band, cm^{-1}; ε_{CT} is the protein molar extinction at the maximum of Amide I band, l mol^{-1} enzyme cm^{-1}; $B_{dioxane}$ is the organic solvent integral molar extinction coefficient, l mol^{-1} cm^{-2}; D_P is the optical density at the maximum of amide I band. The integral absorption extinction coefficient for dioxane ($B_{dioxane}$) was calculated from the area of the absorption band of the pure substance in a cell with a layer thickness of 10 μm: $B_{dioxane}=19{,}100\pm17$ l mol^{-1} cm^{-2}.

The molar absorption extinction coefficient of chymotrypsin was determined measuring the amide I spectra of protein solutions in heavy water. The molar absorption extinction coefficient of chymotrypsin (ε_{CT}) is $80{,}000\pm200$ L mol^{-1} cm^{-1}. The molecular weight of bovine pancreatic α-chymotrypsin was taken as 25,000 Da.

3. Results and discussion

3.1. Dioxane vapor sorption

Fig. 3A shows the dioxane vapor sorption isotherms for solid chymotrypsin. The initial state of the enzyme film (zero hydration level) for studying the sorption of dioxane during hydration (Fig. 3A, curve 1) was obtained by drying in air at water activity less than 0.01 (Film A). On maintaining this "dry" sample in equilibrium with a vacuum of 0.1 Pa and at 25 °C for 3 h, it lost about 0.3% of its weight, which for chymotrypsin implies that at the zero hydration level there are about four water molecules strongly bound to each enzyme molecule. The initial state for studying the sorption of dioxane during dehydration (Fig. 3A, curve 2) was prepared by hydrating the Film A at water activity of 0.98 (Film B). For the second adsorption–desorption cycle (Fig. 3A, curves 3 and 4), the initial state was prepared by dehydrating the Film B in vapors of water-free dioxane (Film C).

As can be seen from Fig. 3A (curves 1 and 2), the adsorption isotherms for the first sorption–desorption cycle show pronounced hysteresis. No sorbed dioxane is observed during hydration at low water activities. Dioxane uptake is markedly increased above water activity of 0.5 and reaches a maximum at water activity of 0.8 (Fig. 3A, curve 1). Dioxane sorption isotherm obtained upon dehydration resembles a smooth curve (Fig 3A, curve 2). At low water activities, chymotrypsin binds about 150 mol dioxane/mol enzyme. These results lend support to the idea that the state of dehydrated enzyme is a non-equilibrium state relative to the sorption of dioxane at low water activities.

A similar behavior was observed for human serum albumin [48]. To generalise this finding, the dioxane

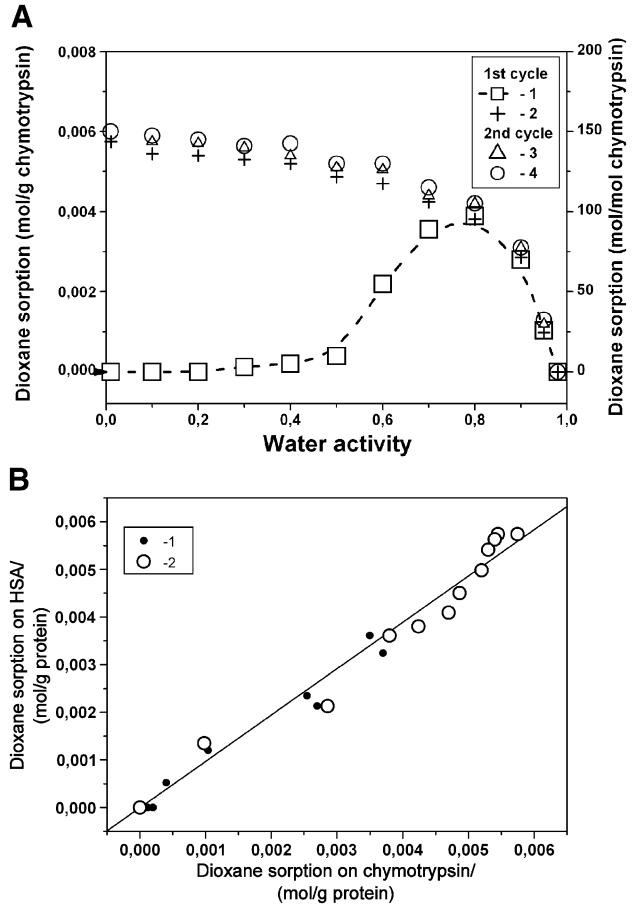


Fig. 3. (A) Sorption of dioxane by α-chymotrypsin as a function of water activity. First adsorption–desorption cycle: (1) Adsorption branch (relative to water sorption). The initial state is Film A from Section 3.1. (2) Desorption branch. The initial state is Film B. Second adsorption–desorption cycle: The initial state is Film C. (3) Adsorption. (4) Desorption. (B) Correlation between the dioxane sorption on human serum albumin A_{dio}(HSA) (modified data from [48]) and dioxane sorption on bovine pancreatic α-chymotrypsin A_{dio}(CT): (1) adsorption and (2) desorption branches (relative to water sorption).

$$A_{dio}(\mathrm{HSA}) = -2.0{*}10^{-5}\left(1.0{*}10^{-4}\right) + 0.97(0.03){*}A_{dio}(\mathrm{CT})$$

Correlation coefficient $R=0.99$; number of experimental points $N=22$; standard error of estimation $s_o=3.2*10^{-4}$.

sorption on human serum albumin was compared with the sorption of dioxane on bovine pancreatic α-chymotrypsin in Fig. 3B. Good linear correlation was observed between the dioxane sorption on HSA and CT (Fig. 3B). This correlation shows that the sorption of dioxane does not depend on the function and secondary structure of a protein. The organic solvent sorption is proportional to the mass of protein sorbent and mainly determined by the protein primary structure. As can be concluded from Table 1, the content of hydrophobic amino acid groups in the primary structure of HSA and α-chymotrypsin is rather close.

For the second adsorption–desorption cycle, when the dehydrated state of solid enzyme is achieved without removing dioxane (Film C), the organic solvent sorption becomes independent of the direction of process and is determined only by the water activity value (Fig. 3A, curves 3 and 4). The second sorption–desorption cycle exhibits no hysteresis within the limits of experimental error (5–10 mol dioxane/mol CT). This means that the dioxane adsorption isotherms started from the high water activity value (a_w~0.98) represent true equilibrium conditions in the whole water activity range. These experimental data are thus accessible to thermodynamic interpretation.

The experimental results reported represent the first example of investigations where the organic solvent sorption–desorption isotherms for solid enzyme were studied over the whole range of water activity.

Table 1
The content of hydrophobic and polar amino acid groups in the primary structure of human serum albumin and bovine pancreatic α-chymotrypsin

Protein	Hydrophobic groups (%)	Polar and ionizable groups (%)
Bovine pancreatic α-chymotrypsin [50]	48.0	52.0
Human serum albumin [49]	41.5	58.5

3.2. Water vapor sorption

Fig. 4 shows the water adsorption and desorption isotherms for chymotrypsin in the presence and absence of dioxane. The initial state of chymotrypsin for studying the water adsorption was a Film A (Section 3.1). The initial state for studying the water desorption was a Film B. For the second adsorption–desorption cycle, the initial state was prepared by dehydrating the Film B in vapors of water-free dioxane (Film C).

As can be seen from Fig. 4, the water sorption isotherms measured in the absence of dioxane resemble typical sigmoidal curves. The water sorption isotherms exhibit

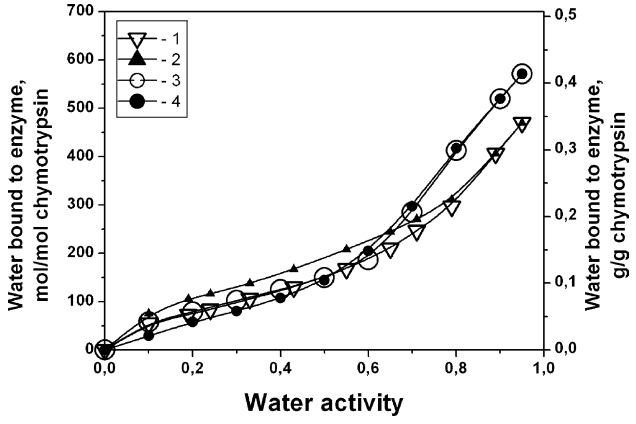


Fig. 4. Water sorption isotherms for α-chymotrypsin. First sorption–desorption cycle in the absence of dioxane: (1) adsorption. The initial state is Film A. (2) Desorption. The initial state is Film B. First sorption–desorption cycle in the presence of dioxane: (3) adsorption. The initial state is Film B. (4) Desorption. The initial state is Film A. Solid lines were fitted by a set of polynomials.

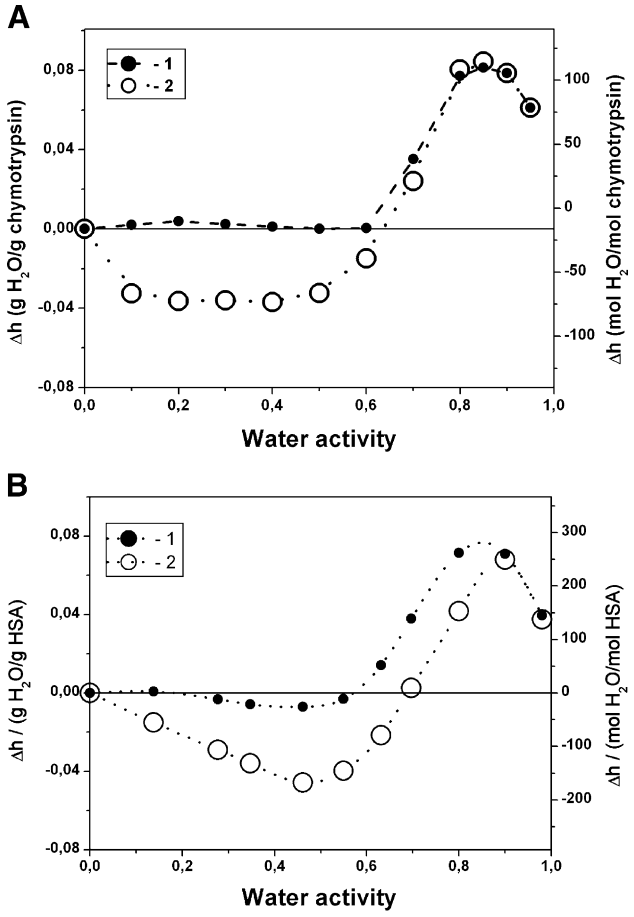


Fig. 5. (A) Bovine pancreatic α-chymotrypsin. Differences in the water uptake in the presence of dioxane: (1) adsorption; (2) desorption. (B) Human serum albumin. Modified data from [48]. Differences in water uptake in the presence of dioxane: (1) adsorption; (2) desorption.

considerable hysteresis (i.e., the water content at a given a_w is higher during desorption than during adsorption).

The presence of organic molecules markedly affects the ability of chymotrypsin to bind water. The effect of dioxane on the water sorption was characterized by the differences (Δh) in water uptake for the corresponding branches in the presence and absence of organic solvent vapor (Fig. 5). Three different effects on water sorption by solid chymotrypsin were observed. At low water activities ($a_w < 0.5$), the water adsorption branch obtained in the presence of organic solvent is similar to that measured in the absence of dioxane (Fig 5A, curve 1). This result is in good agreement with the results reported by Halling [22].

At water activities more than 0.6, both desorption and adsorption branches lie above the corresponding branches for pure water. This result corresponds to the organic solvent-assisted effect on water binding by chymotrypsin.

Upon dehydration in the presence of dioxane, a decrease in the uptake of water was observed at low water activities ($a_w < 0.5$) (Fig 5A, curve 2). This behavior suggests that the suppression in the water uptake is due to a competition for water-binding sites on chymotrypsin by organic molecules. A similar sorption behavior was also observed for HSA (Fig. 5B) [48].

For the second adsorption–desorption cycle, when the dehydrated state of solid enzyme is achieved without removing dioxane (Film C), the water sorption in the presence of organic molecules becomes independent of the direction of process and is determined only by the water activity value (water sorption data for the second cycle are not presented in Fig. 4 for clarity.).

The second sorption–desorption cycle exhibits no hysteresis within the limits of experimental error (0.003–0.005 g water/g CT or 4–7 mol water/mol CT). This means that the water adsorption isotherms measured in the presence of dioxane and started from the high water activity value ($a_w \sim 0.98$) represent true equilibrium conditions in the entire range of water activity. This implies that these experimental data are accessible to thermodynamic interpretation.

These experimental results represent the first example of investigations where the first and second water adsorption–desorption cycles for solid enzyme were studied in the presence of organic solvent over the whole range the water activity.

3.3. Comparison of water and dioxane sorption

Water and dioxane sorption was compared under true equilibrium conditions and at the same values of the thermodynamic activity of sorbate. The dioxane and water sorption isotherms started from the high water activity value (the initial state is Film B) are presented in Fig. 6. As can be concluded from Fig. 6, water sorption is higher than dioxane sorption over the whole range of thermodynamic activity of sorbate. This comparison shows that the interaction of chymotrypsin with H-donating and H-accepting water molecules is more favourable than the interaction of chymotrypsin with dioxane molecules that have no H-donating ability.

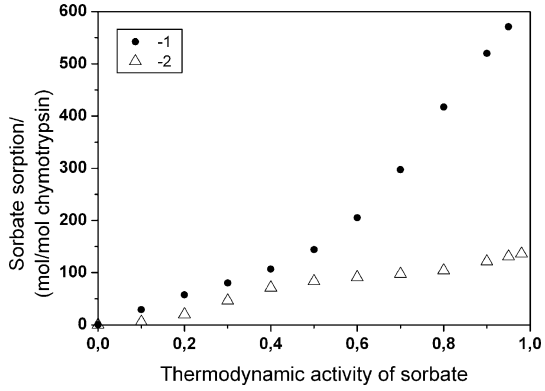


Fig. 6. Water and dioxane sorption by α-chymotrypsin as a function of thermodynamic sorbate activity (first adsorption–desorption cycle. Film B from Section 3.1): (1) water sorption; (2) dioxane sorption.

Table 2
Parameters of water sorption by solid chymotrypsin estimated by Eq. (7)[a]

Type of sorption branch	h_m (mol water/mol enzyme)	K	s_o[b]
In the absence of dioxane			
Desorption [this work]	98.2 (1.9)	23.5 (4.3)	3.3
Desorption [52]	96.7 (2.0)	23.5 (2.2)	3.0
Desorption [53]	103.3 (2.3)	28.5 (6.5)	4.7
Adsorption [this work]	79.3 (1.2)	18.1 (3.0)	1.9
Adsorption [55]	78.4 (0.6)	21.5 (1.4)	3.5
Adsorption [52]	77.4 (0.7)	20.0 (1.7)	2.1
Adsorption [56]	73.3 (3.7)	14.4 (3.7)	4.6
Adsorption [53]	72.3 (1.5)	14.7 (2.2)	5.2
In the presence of dioxane			
Desorption [this work]	97.6 (2.9)	2.2 (0.3)	3.4
Adsorption [this work]	78.5 (1.1)	17.6 (2.6)	2.7

The values of confidence interval of the parameters calculated by Eq. (7) are given in parentheses.
[a] Applicable range (a_w): 0–0.6.
[b] s_o is the residual standard deviation.

3.4. Analysis of water sorption isotherms

The BET model [51] is widely used for describing the sorption ability of various solids, including proteins [1,3,48,52–54] (Eq. (7)):

$$h = h_m \left[\frac{Ka_w}{1 + Ka_w} + \frac{a_w}{1 - a_w} \right] \quad (7)$$

where h is the hydration of solid protein (mol water/mol enzyme); h_m is the number of water binding sites (mol water/mol enzyme); and K is the equilibrium water sorption constant.

It was found that the BET equation (Eq. (7)) describes the isotherms displayed in Fig. 4 up to a water activity no more than 0.6. This result is in close agreement with the data obtained in previous studies on chymotrypsin, serum albumin and other proteins [1,3,48,52–54].

The water sorption parameters estimated from Eq. (7) are presented in Table 2. As can be seen from Table 2, the value of sorption constant K for water desorption branch is markedly smaller in the presence of dioxane than in the absence of organic sorbate. This implies that dioxane molecules suppress markedly the water sorption on solid enzyme at relatively low water activities. No significant organic solvent effect on the value of K was found for water adsorption branch. These results are in agreement with the observations from Sections 3.1 and 3.2.

The values of h_m for the desorption branches as in the absence as well in the presence of dioxane are higher than the similar values for adsorption branches. Most likely this decrease in the h_m values is associated with the lower availability of water binding sites for adsorption process compared with that for desorption one.

Water binding by chymotrypsin was determined in many studies [52,53,55,56]. To confirm the reliability of the obtained sorption data, Eq. (7) was applied to approximate the water sorption isotherms measured by Ruegg [52], Khurgin [53], Adlercreutz [55] and Bone [56]. As can be concluded from Table 2, the results obtained agree well with previously published results.

3.5. Analysis and band assignment of protein infrared spectra

The infrared spectra of Films A, B and C (Sections 3.1–3.3) in the amide I region are presented in Figs. 7 and 9. Fig. 7A and B show the absorbance and second derivative spectra of chymotrypsin in the absence of dioxane in the amide I region. The assignment of individual components to secondary structure was performed as described earlier [28–32]. As can be seen from Fig. 7A and B, the most dominant band component of the chymotrypsin spectra is the band at 1637 cm^{-1}, which is usually attributed to the β-sheet structure [32,34]. The band at 1690 cm^{-1} was assigned to intermolecular β-sheet structure [32]. A minor

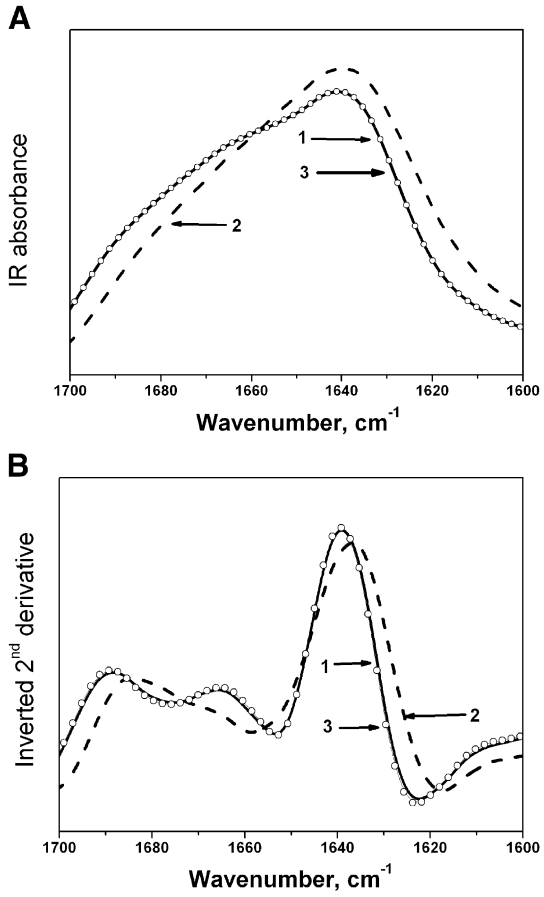


Fig. 7. (A) Absorbance spectra of α-chymotrypsin in the amide I region in the absence of dioxane: (1) Film A from Section 3.1; (2) Film B was prepared by hydrating Film A at water activity of 0.98; (3) Film C was prepared by dehydrating Film B in air at a water activity of 0.01. All the spectra were normalised to the amide I band intensity of a Film A. (B) Second-derivative spectra of chymotrypsin in the amide I region in the absence of dioxane: (1) Film A; (2) Film B; (3) Film C.

component at 1664 cm^{-1} was assigned to irregular secondary structures (β-turns, random coil and extended chains) [36].

As can be seen in Fig. 7A and B, the infrared spectra of chymotrypsin are grossly altered relative to that for the initial dried protein. There are increases in bandwidths and shifts in band positions and relative absorbances, which are indicative of protein conformational changes. This behavior is similar to that noted for chymotrypsin and other proteins [28,30,32].

The hydration–dehydration in the absence of dioxane markedly but reversibly alters the secondary structure of chymotrypsin as revealed by changes in relative absorbance at 1637 cm^{-1} (Fig. 8). At low water activities ($h<0.1$ g/g chymotrypsin), the hydration–dehydration process induces pronounced structural rearrangements. The D_{1637}/D_0 curves as for the hydration as well as for dehydration reach a plateau at water activity above 0.5 ($h>0.1$ g/g chymotrypsin), indicating that the conformational changes are largely completed. These results are in good agreement with the hydration model previously proposed for chymotrypsin [49].

Fig. 9A and B show the absorbance and second derivative spectra of chymotrypsin in the presence of dioxane in the amide I region. As can be seen from Fig. 9A and B, the spectra of chymotrypsin are very different from those in the absence of dioxane. As the water activity was raised from 0.01 to 0.98, the amide I absorbance maxima were red-shifted about 12 cm^{-1} to near 1627 cm^{-1}, indicating the formation of intermolecular β-sheet aggregate [32,57,58]. This broad band at 1627 cm^{-1} in the spectrum of Film B may be caused by the mixing of the native β-sheet band with an intermolecular β-sheet band. As the water activity was decreased from 0.98 to 0.01, the amide I absorbance maximum shifted about 1 cm^{-1} to a higher wavenumber near 1628 cm^{-1}, indicating irreversible conformational rearrangements and the retention of intermolecular β-sheet aggregates. The band at 1628 cm^{-1} is the most dominant feature of the spectrum of Film C (Fig. 9B).

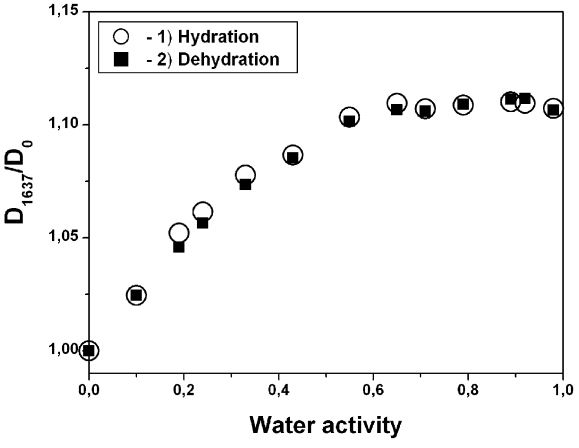


Fig. 8. Absorbance at 1637 cm^{-1} as a function of water activity: (1) adsorption and (2) desorption branches in the absence of dioxane. D_o is the absorbance value at 1637 cm^{-1} for Film A.

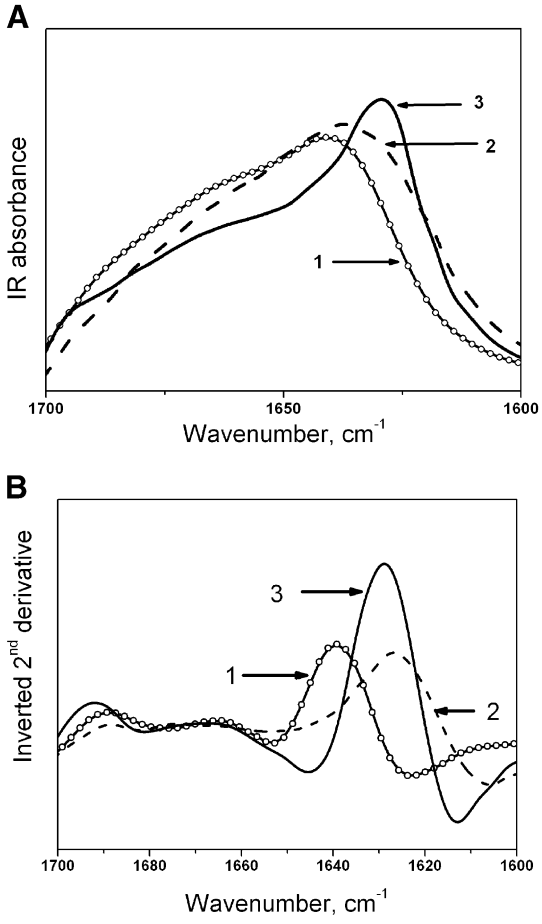


Fig. 9. (A) Absorbance spectra of α-chymotrypsin in the amide I region in the presence of dioxane: (1) Film A from Section 3.1; (2) Film B was prepared by the hydration of Film A in vapors of the water–dioxane mixtures while increasing water activity from 0.01 to 0.98; (3) Film C was prepared by dehydrating Film B by means of dioxane vapor with a water activity of 0.01. All the spectra were normalised to the amide I band intensity of Film A. (B) Second derivative spectra of α-chymotrypsin in the amide I region in the presence of dioxane: (1) Film A; (2) Film B; (3) Film C.

The effect of dioxane on the chymotrypsin structure was characterized by changes in the relative intensity at 1628 cm^{-1} (Fig. 10). The D_{1628}/D_o functions obtained in the presence of dioxane have been compared with those determined for CT in the absence of dioxane. This comparison can show any effects of the organic solvent molecules on the enzyme structure. The differences in the D_{1628}/D_o values obtained in the presence and absence of dioxane are presented in Fig. 11. As can be concluded from Fig. 11, the $\Delta D_{1628}/D_o$ functions are consistent with changes in the dioxane sorption by chymotrypsin (Fig. 3A). Upon hydration, in the water activity range from 0 to 0.5, the $\Delta D_{1628}/D_o$ values are close to zero (Fig. 11, curve 1). Hence, it may be concluded that the presence of dioxane has little effect on the structure of the initially dried enzyme.

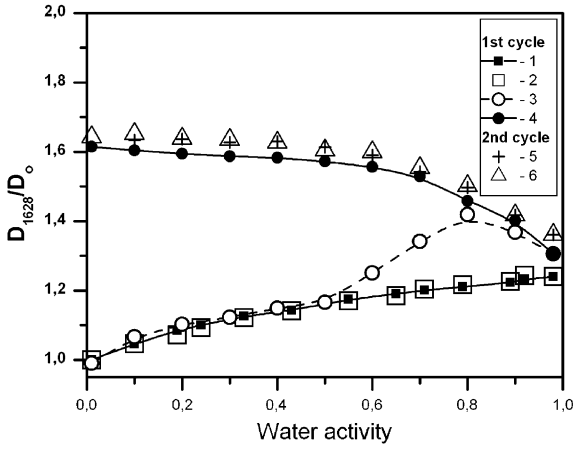


Fig. 10. Absorbance at 1628 cm^{-1} as a function of water activity. First adsorption–desorption cycle in the absence of dioxane: (1) Adsorption. Film A; (2) Desorption. Film B. First adsorption–desorption cycle in the presence of dioxane: (3) Adsorption. Film A; (4) Desorption. Film B. Second adsorption–desorption cycle in the presence of dioxane. The initial state is Film C: (5) Adsorption; (6) Desorption. D_o is the absorbance value at 1628 cm^{-1} for Film A.

Above the threshold water activity of 0.5, the $\Delta D_{1628}/D_o$ values are sharply increased and reach a maximum at water activity of 0.8. There are significant differences between the D_{1628}/D_o values obtained upon dehydration in the presence and the absence of dioxane over the whole range of water activity (Fig. 11, curve 2). These results indicate that the presence of dioxane has a significant effect on the secondary structure of the initially hydrated enzyme.

For the second hydration–dehydration cycle, when the dried state of chymotrypsin is achieved without removing dioxane (Film C), an intensity of the 1628 cm^{-1} band becomes independent of the direction of the process and is determined by the water activity value (Fig. 10, curves 5 and 6).

These results are the first example of investigations where the adsorption–desorption of water and organic solvent on solid enzyme and corresponding changes in the enzyme secondary structure were studied simultaneously over the whole range the water activity.

3.6. Effect of dioxane on the hydration and structure of chymotrypsin

3.6.1. Hydration of chymotrypsin

An explanation of the dioxane effect on the hydration and structure of chymotrypsin may be provided on the basis of earlier hypothesis of water-assisted disruption of the dehydration-induced polar contacts in the solid protein phase [48].

3.6.1.1. Water activity less than 0.5. Like for many proteins, the dehydration of chymotrypsin leads to the formation of protein–protein contacts due to proton-transfer phenomena and hydrogen bonding between polar functional groups. These processes result in a rigid, condensed structure in the dried state. Therefore, certain moieties of the dried protein are unavailable for sorption due to strong interactions between them resulting in sorption and structural hysteresis (Figs. 3, 4, 10).

It was previously shown that the potential of a solvent to form hydrogen bonds is an important factor that controls the state and structure of dehydrated proteins at room temperature [38,39]. Hence, H-donating and H-accepting properties of dioxane are expected to be important for estimating the possible effect of organic molecules on the protein structure and hydration. When a hydrogen bond mediated protein–protein contact is disrupted, sorbate molecules (water or dioxane) may differentiate between H-donating and H-accepting fragments of the disrupted contact. Water (H-donor and H-acceptor) is able to solvate both H-accepting and H-donating groups. An H-accepting dioxane molecule is expected to prefer H-donating groups, while the remaining H-bond of an H-accepting partner will be solvated by water more effectively. Hence, it is expected that dioxane molecules are not effective in disrupting the dehydration-induced protein–protein contacts alone. Therefore, no dioxane sorption (Fig. 3) and dioxane-induced structural rearrangements (Fig. 10) were observed at the lowest water activity values during hydration.

According to this model, by penetrating into the initial dried protein, water molecules hydrate the polar groups of protein–protein contacts and create new sorption sites at the hydrated (disrupted) contacts. Dioxane molecules are unable to compete with water for these new sites at low water activities. This is presumably due to kinetic reasons (steric hindrances and diffusion limitations). For comparison, the molar volume of dioxane is 85.2 cm^3/mol. In contrast to dioxane, the molar volume of water is 18 cm^3/mol. Therefore, no decrease in water sorption (in comparison with that for pure water) was observed in

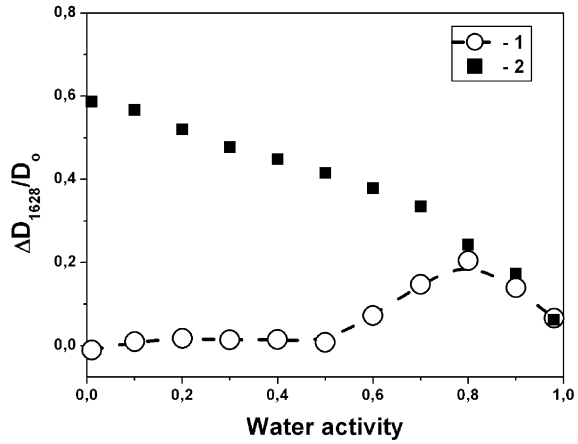


Fig. 11. Differences in the absorbance at 1628 cm^{-1} as a function of water activity. First sorption–desorption cycle: (1) Adsorption. Film A. (2) Desorption. Film B.

the water activity range from 0 to 0.5 during hydration (Fig. 5A and B, curves 1).

3.6.1.2. Water activity more than 0.5. The penetration of dioxane molecules occurs together with the hydration and disruption of most of these dehydration-induced protein–protein contacts ($a_w > 0.5$; $h > 0.1$ g/g). The sharp increase in dioxane uptake at water activity above 0.5 (Fig. 3A, curve 1) provides an example of the hydration-assisted effect on dioxane sorption.

There is a tradeoff between the hydration-assisted effect on dioxane sorption versus water/dioxane competition for new sorption sites at the disrupted contacts. The interplay between the hydration-assisted effect and water/dioxane competition may be interpreted from the maximum in dioxane sorption at $a_w = 0.8$ (Fig. 3).

From the other hand, each dioxane molecule contains four hydrophobic CH_2 groups. Therefore, it is expected that by penetrating into the hydrated protein ($a_w > 0.5$; $h > 0.1$ g/g), dioxane molecules (in contrast to water) are able to solvate polar moieties not only in hydrophilic but also in hydrophobic regions of the protein creating new sites for water sorption. The additional water uptake that was observed at high water activities (Fig. 5A and B) is an example of the dioxane-assisted effect on water binding by chymotrypsin.

Calorimetric heat effects of the interaction of solid protein with the water–dioxane mixtures were also sensitive to changes in the mechanism of water sorption. The additional hydration of HSA at water activity in dioxane about 0.6 was accompanied by a big exothermic drop of the heat effects [24,25,48].

3.6.2. Dehydration of chymotrypsin

The presence of dioxane has a significant effect on the enzyme hydration and structure in the water activity range from 0 to 0.98. The number of available water binding sites is higher during dehydration than during hydration as revealed from the BET monolayer values (Table 2). This is due to the disruption of the protein–protein contacts in the initially dried enzyme and the creation of new sorption sites. All these water binding sites are available to the interaction with dioxane molecules. There are no steric limitations for dioxane sorption on these water binding sites during dehydration.

There is a compromise between the dioxane-assisted effect on water sorption and water/dioxane competition for new sorption sites at the disrupted contacts. The difference curve 2 (Fig. 5A) provides an example of the change in dominance between the dioxane-assisted effect at high relative water activities versus competition at low relative water activities.

The dioxane-induced conformational rearrangements for chymotrypsin (Fig. 11) appear to be due to the compensation for the loss of hydrogen bonding with water molecules during dehydration. At high water activities, hydrogen bonds of ionizable and polar protein groups are satisfied by water molecules. Upon dehydration, these hydrogen bonds are lost. To compensate this loss, the enzyme rearranges its conformation to maximize intra- and inter-protein hydrogen bonding with the dioxane molecules to replace lost hydrogen bonds to water. This resulted in observed intermolecular β-sheet conformation (Fig. 9). The penetration of dioxane molecules during dehydration into the solid enzyme and the formation of additional rigid elements in the secondary structure prevent the creation of the dehydration-induced protein–protein contacts, which are responsible for sorption and structural hysteresis.

3.7. Practical implications of the data obtained in the context of nonaqueous enzymology

On the basis of the sorption and structural results reported the effect of hydration history on the enzymatic activity-a_w profile may be classified. Since the effect of hydration history may be overlapped by the effect of chemical modification, this classification does not include the chemically modified enzyme preparations. According to this model, the profile of catalytic activity against a_w will be determined by two effects: (a) the dehydration-induced protein–protein contacts (described by the $\Delta D_{1637}/D_o$ function, Fig. 8) and (b) the organic solvent-induced intermolecular aggregation (described by the $\Delta D_{1628}/D_o$ function, Fig. 11).

This classification includes the following types:

Type 1. The initial state is the enzyme with high water content ($h \sim 0.3$–0.4 g/g) in equilibrium with water–organic mixture. The structural and sorption behavior of this preparation will be similar to that noted for Film B. It is expected that the catalytic activity-a_w profile will be determined by the organic solvent sorption and organic solvent-induced conformational rearrangements (Fig. 12, curve 1). No attempt was made to study the enzymatic activity in this case.

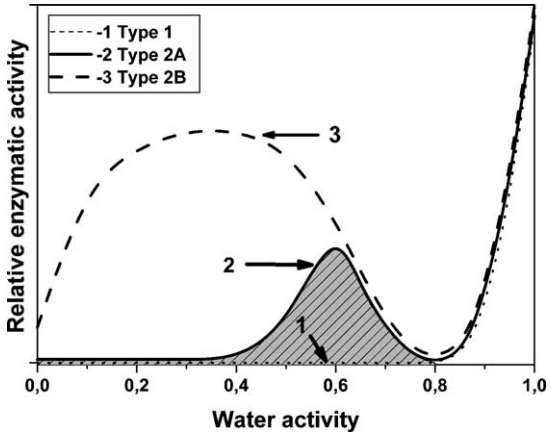


Fig. 12. The enzymatic activity-a_w profiles as a function of the enzyme hydration history: (1) Type 1; (2) Type 2A; (3) Type 2B.

Type 2A. The initial state of this enzyme preparation is the initially dehydrated enzyme. The water content of the enzyme is lower than 0.1 g/g. The structural and sorption behavior of this preparation will be similar to that noted for Film A.

Typical examples are given in Fig. 13A (curve 3) and Fig. 13B (curves 1 and 2). No enzymatic activity was observed at low water activities (Fig. 13B, curves 1 and 2). In this water activity range, the dehydration-induced contacts play a negative role distorting the active enzyme conformation and creating steric hindrances. A minimum in the catalytic activity was observed at a_w of 0.8 in dioxane (Fig. 13A, curve 3). This minimum correlates well with the position of the maximum in dioxane sorption (Fig. 3, curve 1) and organic solvent-induced intermolecular aggregation (Fig. 11, curve 1).

For comparison, Fig. 13A (curve 1) shows how a_w affects the catalytic activity of chymotrypsin in the absence of organic solvent. As can be seen from Fig. 13A (curve 1), no enzymatic activity was observed at low a_w values ($a_w<0.5$) due to the dehydration-induced contacts. At $a_w>0.5$, the catalytic activity is sharply increased reaching a maximal value at $a_w\sim0.7$. It is expected that in this water activity range ($a_w=0.5$–0.7), a fraction of the dehydration-induced contacts is decreased to zero. No minimum in catalytic activity was observed at an a_w of approximately 0.8 in the absence of organic solvent.

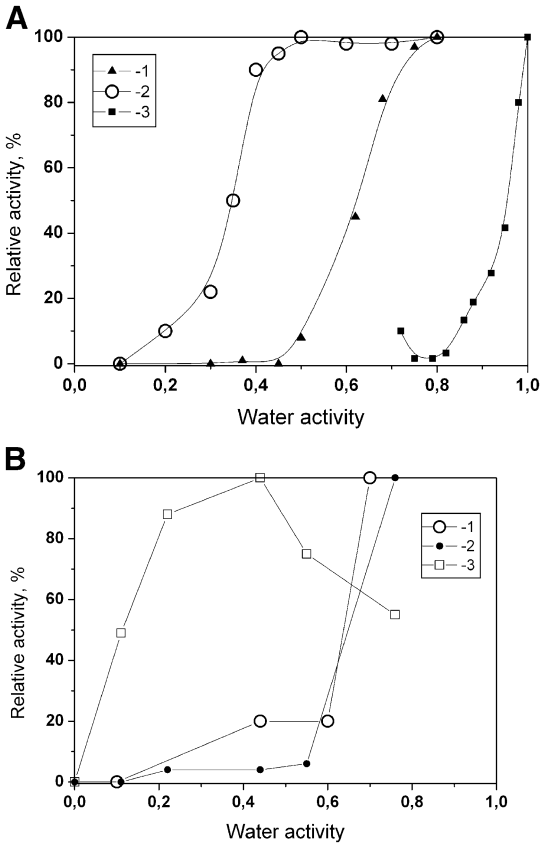


Fig. 13. (A) Profiles of catalytic activity as a function of a_w for the chymotrypsin-catalysed hydrolytic reactions: (1) Solid-state solvent-free hydrolysis of *N*-succinyl-L-phenylalanine-*p*-nitroanilide [59]. (2) Solid-state solvent-free hydrolysis of *N*-succinyl-L-phenylalanine-*p*-nitroanilide in the presence of sodium acetate (17%, w/w) [59]. (3) Hydrolysis of *N*-acetyl-L-tyrosine ethyl ester in water–dioxane mixtures. Modified data from [13]. Catalytic activities were normalised relative to the maximal value. (B) Profiles of enzymatic activity as a function of a_w for the enzyme-catalysed reactions in organic solvents: (1) Chymotrypsin-catalysed transesterification in hexane. Modified data from [60]. Transesterification reaction in acetonitrile catalysed by freeze dried (2) and propanol-1 dehydrated (3) subtilisin Carlsberg. A similar catalytic behavior was observed for α-chymotrypsin [61]. Catalytic activities were normalised relative to the maximal value.

As a result, the profile of catalytic activity–water activity in the organic solvent may be qualitatively described by curve 2 (Fig. 12) with a peak at the water activity value of approximately 0.6 (Fig. 12, curve 2). At an a_w of 0.5–0.7, the residual dehydration-induced contacts play a positive role preventing the organic solvent-induced aggregation. The difference in enzymatic activity between Types 1 and 2A is shown shaded (Fig. 12).

Type 2B. This type of enzyme pretreatment includes the dehydration with an activating additive as one of the preparation steps. In relation to the hydration–dehydration process, Type 2B may be considered as an intermediate situation between Type 1 and Type 2A. Typical examples of this type of enzyme pretreatment are the following:

a) Co-lyophilisation with some additives (for example, salts (Fig. 13A, curve 2), crown ethers, and cyclodextrines);
b) Dehydration by washing with organic solvent (propanol-1 or acetonitrile) (Fig. 13B, curve 3).

The catalytic activity at low water activities for Type 2B (Fig. 12, curve 3) may be explained by two effects:

(A) A significant part of the dehydration-induced protein–protein contacts is not formed due to the dehydration in the presence of additive. This results in increase of the proportion of the enzyme molecules in a conformation close to the active form (Fig. 13A, curve 2, and Fig. 13B, curve 3).
(B) Residual dehydration-induced contacts after the pretreatment with activating agent play a positive role in preventing the organic solvent-induced intermolecular aggregation. As shown in Sections 3.1 and 3.5, no organic solvent sorption and organic solvent-induced structural rearrangements were observed at the lowest water activity values during hydration.

4. Conclusions

– A new experimental approach based on FTIR spectroscopic measurements provides an informative tool in monitoring the molecular processes that occur on the hydration–dehydration of solid enzyme in the presence of organic solvent.

– The results presented demonstrate that the hydration and structure of α-chymotrypsin depend markedly on how enzyme has been hydrated — whether in the presence or in the absence of organic solvent.
– The results obtained show that the dehydration-induced protein–protein contacts are one of the important factors that determine the enzymatic activity-a_w profile. Understanding of the relationships between the activity and stability of the enzyme-low molecular additive complexes and molecular structure of activating additives is one of the promising ways for the preparation of effective biocatalytic systems in nonaqueous media.

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