ELSEVIER

Biophysical Chemistry 81 (1999) 107–123

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Supramolecular interactions of solid human serum albumin with binary mixtures of solvent vapors

V.V. Gorbatchuk*, M.A. Ziganshin, B.N. Solomonov

Department of Chemistry, Kazan State University, Kremlevskaja 18, Kazan, 420008, Russia

Received 8 January 1999; received in revised form 25 May 1999; accepted 24 June 1999

Abstract

Sorption isotherms of organic compounds on solid human serum albumin (HSA) from binary vapor mixtures were determined by gas chromatographic headspace analysis. The shape of sorption isotherms depends on molecular structure of studied sorbates. The 'active' compounds capable to sorb effectively on dry HSA increase the sorption of 'passive' compounds unable to be sorbed by dry HSA in absence of the third component. The critical hydration of HSA is required for sorption activation of 'passive' sorbates if water is taken as 'active' component. Ethanol and acetonitrile exhibit such activation effect without threshold. 'Passive' sorbates are able to produce cooperative activation effect on the sorption of 'active' component. Hydration history effect is observed for sorption on prehydrated HSA and HSA hydrated in situ. Obtained results were interpreted in terms of clathrate formation by 'passive' sorbate (substrate) and 'active' component inside the HSA (receptor) binding centers.

Keywords: Substrate binding; Sorption cooperativity; Protein hydration; Hydration history effect; Clathrate formation

1. Introduction

Molecular interactions of proteins with organic substances as substrates with formation of 'supermolecules' are generally recognized to play important role in enzymatic reactions, transport phenomena and antigen–antibody interactions [1]. Application of organic compounds as solvents for enzymatic reactions has drawn attention to the problem of molecular interactions between organic substances and proteins in a wider aspect. Organic solvents were found to produce a number of effects on enzymatic activity and receptor properties of the protein [2–5]. Except the participation in the direct protein–solvent interactions the organic solvent may have influence on the

* Corresponding author.
E-mail address: valery.gorbatchuk@ksu.ru (V.V. Gorbatchuk)

0301-4622/99/$ - see front matter
PII: S0301-4622(99)00087-3

protein hydration at the fixed water activity [3,4,6–10]. Change of the hydration level at some critical value is usually accompanied by the change of the protein properties: conformational mobility [2,11–13], and dynamical glasslike transitions [13]. Thus the bound water is regarded as essential structural element of the protein receptor system. Some organic solvents also can partially play the role of water in the protein–substrate interaction [14,15]. Consequently the problem of the classification of the molecular interactions between proteins and organic substances is the problem of choice between substrate and water-like functions of the studied compound. Obviously any combination of these two functions can not be excluded in view of variety of molecular structure of organic compounds. For example, ethanol is regarded as able to partially replace water in the enzymatic reactions of immobilized laccase [15]. In the other reaction of catalyzed by alcohol oxidase gas-phase oxidation the ethanol is a substrate [16]. Besides, the situation without significant binding and without substantial solvent effect on the protein binding properties is also possible. It may take place in low-water media where enzymes usually demonstrate very poor activity [2,4]. In our previous study [17] dried human serum albumin (HSA) with 0.008 g H_2O/g protein was shown to have strong dependence of sorption properties on the molar volume of sorbate that can not be explained by the sorption on fixed surface. The sorption affinity of dried HSA to sorbates with small monofunctional molecules (methanol, ethanol, acetonitrile, nitromethane) is comparable with the sorption affinity to water but sharply falls with the increase of the sorbate molecular size. The sorption of 2-propanol and pyridine by dried HSA is very small [17]. However, HSA in water solutions is able to bind a numerous quantity of substrates with large molecules including hydrophobic substances [18].

The dry protein and its water solution constitute the extreme points of the protein hydration coordinate. The hydration effect on the sorption of organic compounds at the intermediate water activity values in the ternary systems protein + organic component + water is practically not studied. The effect of the organic component of the media can be found in the observed stepwise increase of water uptake on sorption isotherms for HSA suspended in water/dioxane and water/*n*-butanol mixtures at high protein hydration levels [7,9,10], and in the higher water uptake values for chymotrypsinogen and alcohol oxidase suspended in some water/organic mixtures than at water sorption from air [6]. But these results do not allow us to make definite conclusions about the possibility of simultaneous sorption increase of organic component by suspended protein. Nevertheless, such a situation has been considered to be possible [6]. Complete investigation of this problem requires the independent variation of activity of all sorbates in the system. It is not suitable in the protein solutions or suspensions in water/organic mixtures where organic component of liquid phase is in large excess compared to its uptake by the protein.

In the present study we have examined the mutual influence of volatile sorbates in binary vapor mixtures on their vapor sorption on the solid human serum albumin in absence of liquid phase.

2. Experimental

All measurements were carried out on human serum albumin ('Reanal', Hungary, product N 01092, lyophilized, with electrophoretic purity $>$ 95%, remainder after burning $<2\%$). Total concentration 0.2% of fatty acids (C_{12}–C_{22}) in this protein preparation was determined by usual technique [19], that includes extraction by chloroform–methanol (2:1) mixture, the washing of extract by water, methylation with diazomethane in diethyl ether and GC determination in the concentrated *n*-hexane solution. Organic compounds were of the reagent grade qualification (purity $>99\%$) and were dried by the standard methods [20] before the experiment.

The HSA preparations with constant hydration were prepared by air hydration of dried HSA samples (0.300 g, hydration 0.01 $h = 0.01$ g H_2O/g dry HSA). Samples of HSA in 15-ml open vials were placed in a dessicator with water at the bottom and were held at room temperature, 295

K, for 36 h. The added amount of water was calculated as necessary to reach the desired hydration of the protein, taking into account the initial HSA hydration and air humidity. All added water was evaporated in the first 10 h. Then the liquid organic compound (sorbate) was carefully dosed with a microsyringe on the internal walls of the vials in order to avoid direct contact between liquid sorbate and the solid protein. The volume of the added liquid was in the range 1–20 μl, depending on the organic compounds. Immediately afterwards the vials were sealed with fluoroplaste (0.2 mm) and silicon linings and were held at 298 K for 72 h. One of the vials with HSA sample hydrated in the same pot and treated in the same way but without organic sorbate was taken for determination of the HSA hydration. Hydration of HSA preparation was determined from a loss of weight at 298 K and pressure below 1 Pa with a microthermoanalyzer MGDTD-17S (SETARAM) as g water/g dry HSA. The drying was continued until the weight of albumin sample (~10 mg) ceased to vary for 5–6 h within 0.1%. The vial where drying was performed has the volume ~100 ml. The estimated error of HSA hydration determination is 0.002 h.

Hydration of HSA samples by the second method was performed in situ through air saturation by liquid mixtures of organic sorbate (6 vol.%) and water (94 vol.%). Of this solution 1–50 μl was dosed in open little glass containers placed in the 15-ml vials with 0.300 g dried HSA (0.01 h) to prevent direct contact between liquid and protein preparation during equilibration. Water and benzene were mixed just in these containers within the vials. The vials were then sealed as mentioned above and held at 298 K for 72 h before sorption isotherm determination. The HSA hydration was calculated by added quantity of water, initial humidity of air in the vial and initial hydration of HSA preparation assuming that protein-vapor distribution of water corresponds to data from Bull [21] for horse serum albumin. The water sorption on HSA suspensions in binary water mixtures with acetonitrile, ethanol, 1-propanol and dioxane and other organic solvents was shown [7–10] to be at least the same or higher than calculated by the sorption isotherm in the binary system water + protein. According to vapor sorption data in binary water–protein systems [21] at least 99.5% of water in a hermetically closed 15-ml vial with 0.300 g dried HSA must be sorbed by protein preparation if the total quantity of water in the vial is 4–50 mg. We checked the applicability of this conclusion to the ternary systems studied in the present work. For each studied organic sorbate three ternary systems with different quantities of water–organic liquid mixture (6 vol.% organic sorbate + 94 vol.% water) were prepared and equilibrated in the same way as for headspace analysis. The quantity of sorbed material on HSA was determined gravimetrically by loss of weight as described above. The loss of weight of equilibrated protein samples corresponds to a sum of weight of added liquid and residual water amount within ±4% for samples with high final hydration (0.13–0.15 h) and ±10% for samples with low final hydration (0.02–0.06 h). The corresponding error of HSA hydration estimation is ±0.007 h. Significant part of this error is due to the error of liquid dosing into the vial (1–1.5%), to equilibration losses (0.1–1% of the added liquid) and to the error of gravimetrical experiment (±0.002 h).

The systems of dried HSA (hydration 0.01 h) + two organic sorbates were prepared by the same technique as for saturation by water solutions.

Vapor sorption isotherms of organic sorbates were determined by gas chromatographic (GC) headspace analysis. Automated headspace dosing system of original design [22] was used to dose the vapor phase from the sealed vial in a capillary chromatographic column. In this sampler a principle of electropneumatic dosing, described in [23], is applied. The dosing system does not contain any metal or heated elements, with which the vapor sample could contact on the way from a vial to the chromatographic column. The total volume of all connecting paths on this way is less than 30 μl. Thus, distortions caused by the sorption on internal parts of the dosing system were avoided. Fused silica chromatographic column (25 m × 0.25 mm i.d., SE-54) and the flame ionization detector were used in the analysis.

The activity of sorbate (P/P_0) was determined as a ratio of the area of its chromatographic

peaks for vapor phase over HSA and over sorbate pure liquid at 298 K. Absence of overlaid impurities' peaks was checked by comparing the height/area ratio for peaks of sorbate over protein and over pure liquid. In the majority of cases the sorbate activity ceased to vary after the first 24 h after a beginning of equilibration. The errors of the activity determination were in the range from 5% for sorbate activities over 0.5 to 10% for activities below 0.1.

Uptake V_S of sorbate on HSA was calculated from the difference between the total amount of a sorbate in the system and its amount in the vapor phase. The last value was calculated by measured activity (P/P_0), saturated vapor pressure at 298 K taken from the literature [24,25], and the volume of vapor phase of the vial. The isotherms were corrected on the sorbate loss at equilibration that was estimated in blank experiments without HSA preparation. This loss is equal to 0.1–1% of the total sorbate amount in the vial for alcohols and acetonitrile depending on their activity and 1% for other sorbates at high HSA hydration values or ethanol activity. Maximum losses for dioxane, ethylacetate and benzene are 8% in systems with HSA hydration below 0.08 h where the most part of the added organic sorbate remains in vapor phase after equilibration. Estimated error of V_S determination is 5%. No volatile organic impurities were detected in the headspace over the HSA samples in the vapor sorption experiments.

The limiting activity coefficients γ^∞ were determined at 298 K with precision $\pm 10\%$ by the same headspace technique for infinitely dilute solutions (0.2 vol.% for solution of ethanol in benzene and 1 vol.% for other solutions). Absence of the concentration dependence of γ^∞ values was tested in each case. As distinct from the samples with solid HSA the equilibrium in hermetically closed 15-ml vials with 1 ml liquid solution is reached in several minutes. Equilibration process was controlled by the chromatographic peak height/area of the solute in subsequent cycles of headspace dosing and GC analysis for the same vial with solution. Correction on the redistribution of some part of the solute to the vapor phase of the vial was made in each case. The theory of determination of limiting activity coefficients by GC headspace analysis was described earlier [26].

3. Results

The choice of studied sorbates is based on their ability to be sorbed by dry HSA. We studied the influence of 'active' substances (water, ethanol and acetonitrile) on the sorption of 'passive' compounds (dioxane, ethylacetate and benzene). The term 'active' is used for description of high sorbate ability to be sorbed by dry HSA. The effective BET 'monolayer' volumes of 'active' sorbates (acetonitrile and ethanol) for dry HSA (0.008 h) [17] correspond to 40 and 27%, respectively, of the water monolayer volume (activity range 0.05–0.4) for horse serum albumin [21]. The term 'passive' describes the absence of the sorbate sorption exceeding the sensitivity limit of the applied headspace technique. In the present work we have not observed any uptake of benzene, ethylacetate and dioxane by dry HSA (0.01 h) at sorbate activities 0.5–0.8. 1-Propanol and 2-propanol were studied as intermediate substances. Their sorption on dry HSA is small but perceptible [17]. In addition, the influence of HSA hydration on the sorption of 'active' substances (ethanol and acetonitrile) was studied.

Influence of the hydration HSA on the sorption of organic compounds was studied for prehydrated HSA with constant hydration and for HSA hydrated in situ with varied water contents. Respectively, the two types of sorption isotherms were determined. The sorption isotherms of the first type (Figs. 1–3) are the dependencies of the sorbate uptake on the sorbate activity for the prehydrated HSA with constant hydration. Parameters of sorption isotherms are presented in Table 1. Along with effective BET monolayer volume V_m and sorption constant C the ratio $[V_S/(P/P_0)]_{6\%}$ was calculated by BET equation as the $V_S/(P/P_0)$ value at 6 vol.% of organic sorbate and 94 vol.% water of the liquid mixture added to the dried HSA (0.01 h). The value of $[V_S/(P/P_0)]_{6\%}$ is necessary for comparison of the results of used methods of the sorption isotherm

determination. In the cases where BET approximation fails to provide stable solutions the average isotherm slope $V_S/(P/P_0)$ at zero intercept was calculated. Most of the obtained isotherms for prehydrated HSA (Figs. 1–3) are approximately linear. Only isotherms determined for dried HSA (0.01 h) have a large curvature (Figs. 2 and 3, [17]). Analogous linear sorption isotherms were observed earlier for sorption of aromatic hydrocarbons on collagen from water solution [29]. The near-to-linear shape of obtained isotherms for hydrated HSA shows a weak dependence of the binding affinity of HSA to the studied sorbate on its activity. But the hydration of HSA is a very essential factor that obviously determines the slope of the sorption isotherms. Increase of HSA hydration produces the increase of the isotherm slope for 1-propanol, 2-propanol and dioxane. The isotherm slope of acetonitrile is reduced upon the HSA hydration (Fig. 3).

For determination of the isotherms of the second type the binary liquid mixture of sorbates with the constant component ratio was used as the source of vapors of two sorbates in the system. The concentration of the minor component (6 vol.%) in this mixture was chosen to be low enough to diminish its influence on the vapor sorption of the major component on HSA. On the other hand, the concentration of the minor component must be sufficient to provide its relatively large activity without existence of liquid phase in the system at high quantities of the added mixture (< 50 μl/0.300 g HSA) after equilibration. Varied contents of both sorbates in the system makes it possible to analyze their mutual influence on the binding affinity of HSA to each

Table 1
Parameters of vapor sorption isotherms for prehydrated HSA at 298 K

Sorbate	HSA hydration (h)	V_m (μl/g)	C	$V_S/(P/P_0)_{6\%}$ [a] (μl/g)
Acetonitrile	0.008[b]	28.0 ± 2.7[c]	16.8 ± 3.4[c]	470[e]
	0.01	46 ± 1[d]	10.4 ± 0.8[d]	474[f]
	0.029	28 ± 1[c]	7.3 ± 0.6[c]	199[e]
	0.122	17.2 ± 2.7[c]	6.4 ± 2.8[c]	81[e]
Dioxane	0.108	–	–	7.5[g]
	0.115	12.5 ± 4.3[c]	3.0 ± 1.9[c]	31.8[e]
	0.156	37 ± 1[c]	5.8 ± 0.5[c]	197[e]
1-Propanol	0.008[h]	11.1 ± 0.8[c]	12.9 ± 4.5[c]	143[e]
	0.101	–	–	194[g]
	0.155	61 ± 18[c]	4.3 ± 2.1[c]	248[e]
2-Propanol	0.008[b]	1.1 ± 0.1[c]	–	4.2[g]
	0.101	16.5 ± 0.8[c]	6.1 ± 1.4[c]	86[e]
	0.155	91 ± 20[c]	3.2 ± 1.0[c]	277[e]

[a] $V_S/(P/P_0)$ value calculated by BET equation at volume ratio 6:94 of total quantity of liquid organic component and water added to dried HSA (0.01 h).
[b] Data from ref. [17].
[c] Approximation parameters of BET equation [27].
[d] Approximation parameters of Langmuir equation.
[e] Initial slope of the BET isotherm at $(P/P_0) = 0$.
[f] Initial slope of the Langmuir isotherm at $(P/P_0) = 0$.
[g] Average slope of the sorption isotherm $V_S/(P/P_0)$ at zero intercept.
[h] Data from ref. [28].

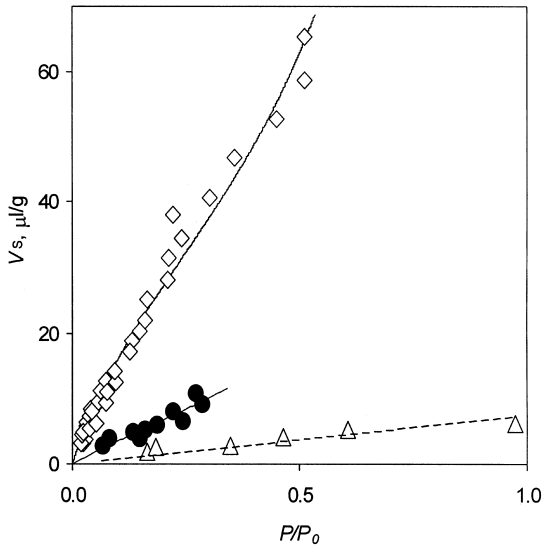

Fig. 1. Vapor sorption isotherms of dioxane on prehydrated HSA at 298 K at fixed hydration: △, 0.108 h; ●, 0.115 h; ◇, 0.156 h. Solid lines are BET isotherms; dashed line is linear trend with zero intercept.

sorbate in the system except water. For water, only its contents in solid phase at equilibrium were calculated (see Section 2) but not activity. The sorption isotherms of the second type are presented as the plots of the sorbate uptake/activity ratio $V_S/(P/P_0)$ vs. the HSA hydration or activity of the second organic sorbate (Figs. 4–6). Full data are given in Tables A1–A7 in Appendix A. The value of sorbate uptake per unit of its activity $V_S/(P/P_0)$ is chosen as a sorption property to minimize the quantity of the system varied parameters that remain outside of the two-dimensional isotherm presentation for the ternary system. The linear shape of sorption isotherms for prehydrated HSA (Figs. 1–3) shows that the value of $V_S/(P/P_0)$ is approximately constant in the relatively large interval of sorbate activity at the constant hydration of HSA. So the value of $V_S/(P/P_0)$ may be assumed to be an appropriate parameter of HSA sorption affinity to studied sorbate at the given HSA hydration. The same approach was used for investigation of the ternary systems of HSA + two organic sorbates with one sorbate being 'active' in the sense mentioned above. On the other hand, the isotherms with $V_S/(P/P_0)$ as a sorption property plotted vs. the HSA hydration can be compared with the results of investigations of enzyme hydration influence on the rates of enzymatic reactions in gas phase reactors [16,30]. The results of these studies have been presented as the dependence of the kinetic parameters of the enzymatic reactions on the enzyme hydration at the constant activity of reagents.

The sorption isotherms in Figs. 4 and 5 demonstrate the cooperative increase of binding affinity $V_S/(P/P_0)$ above the threshold or critical value of HSA hydration for the studied 'passive' sorbates (benzene, ethylacetate and dioxane) and for 'intermediate' sorbates (1-propanol and 2-propanol). The critical hydration values for these systems are given in Table 2. Propanols have significantly lower critical values of HSA hydration than 'passive' sorbates. At further increase of HSA hydration the $V_S/(P/P_0)$ value reaches some saturation level that is specific for each studied organic sorbate. For propanols the slight decrease of the $V_S/(P/P_0)$ value at high HSA hydration is observed. The saturation value of

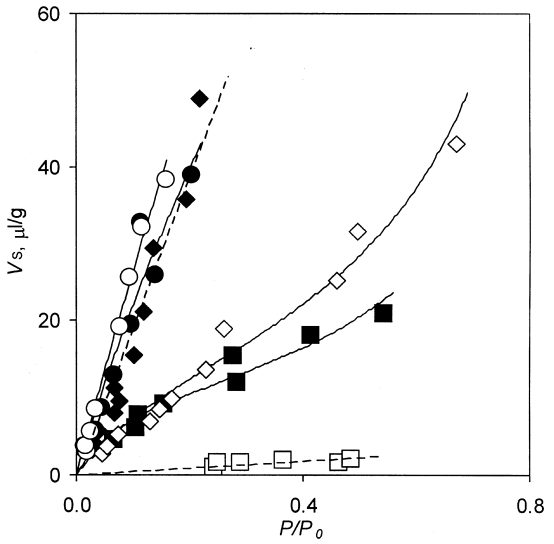

Fig. 2. Vapor sorption isotherms of 1-propanol and 2-propanol on prehydrated HSA at 298 K at fixed hydration: ■,□, 0.008 h (data from [17]); ◆,◇, 0.101 h; ●,○, 0.155 h. Empty points correspond to 2-propanol, filled points correspond to 1-propanol. Solid lines are BET isotherms; dashed line is linear trend with zero intercept.

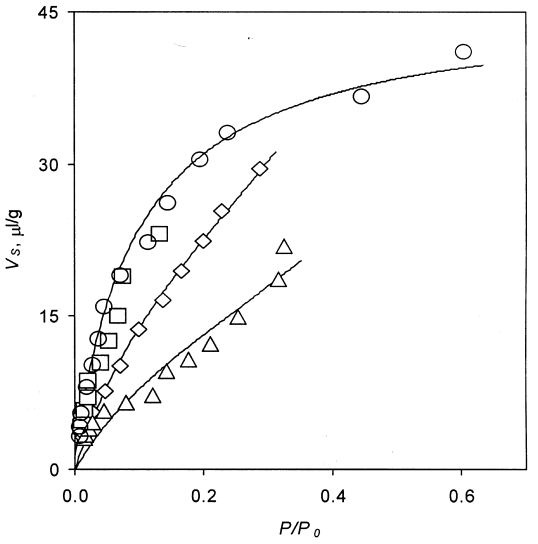

Fig. 3. Vapor sorption isotherms of acetonitrile on prehydrated HSA at 298 K at fixed hydration: □, 0.008 h (data from ref. [17]); ○, 0.01 h; ◇, 0.029 h; △, 0.122 h. Solid lines are Langmuir isotherm (0.01 h) and BET isotherms (0.029 h and 0.122 h).

HSA sorption affinity to benzene $V_S/(P/P_0)_{sat}$ = 69 μl/g obtained in the present work practically coincides with the benzene binding capacity of HSA in water solution (56 mol/mol HSA or 75 μl/g [32]). This fact is in line with the conclusion that no major hydration-induced conformational changes occur in the protein above hydration of approximately 0.2 h [13]. A cooperative hydration effect analogous to the effect observed in the present work was found for enzymatic activity above the critical hydration for enzymes in gas-phase reactors [16,30] at the fixed activity of substrates. The same kind of hydration influence was observed also in solid phase enzymatic reactions [33,34], and for enzymatic reactions in water/organic mixtures [4,13,15,35] where organic component of the solvent has approximately constant activity $P/P_0 \approx 1$ being in large excess.

The dependence of the $V_S/(P/P_0)$ value for ethanol and acetonitrile on HSA hydration (Fig. 6) has essentially a different shape than for 'passive' sorbates. The decrease of $V_S/(P/P_0)$ value is observed up to HSA hydration ~ 0.06 h for ethanol and ~ 0.09 h for acetonitrile. The shape of sorption isotherms in Fig. 6 may be the result of two effects: (1) concurrence between water and 'active' sorbate for sorption sites of HSA (Fig. 3); and (2) non-linearity of sorption isotherms in their ordinary presentation (V_S vs. P/P_0) for 'active' sorbates at fixed HSA hydration on dried HSA (see Fig. 3, [17]). As can be seen from Table A7 in Appendix A the V_S values for acetonitrile sorbed by wet HSA are lower than the V_S values interpolated on the basis of sorption isotherms for dried HSA (0.01 h) approximated by Langmure equation with parameters from Table 1. There is no significant difference in V_S values for ethanol sorbed by wet and dried HSA (Table A6). For 'intermediate' sorbate 1-propanol the HSA hydration leads to the increase of sorption (Table A1). The different effects of organic components classified here as 'passive' and 'active' were observed earlier for enthalpies ΔH of prehydrated HSA (0.1 h) immersion in water/organic mixtures [10]. A cooperative effect of the 'passive' (dioxane, pyridine, n-butanol) and 'intermediate' (1-propanol) component of the solvent on ΔH values takes place above the critical water activity

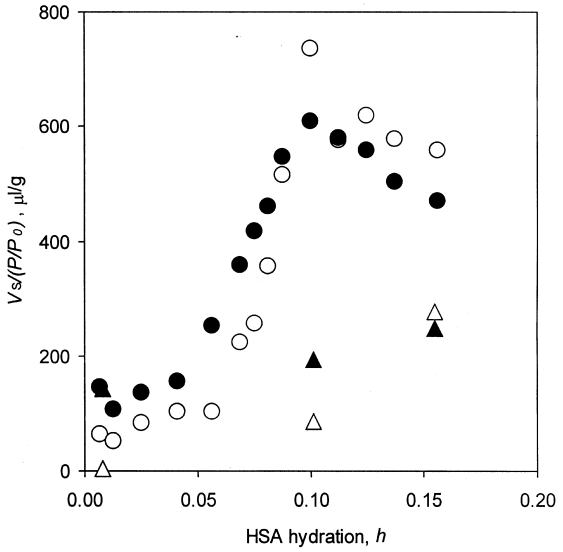

Fig. 4. Sorption isotherms of 1-propanol (●) and 2-propanol (○) on solid HSA (0.01 h initially) equilibrated at 298 K with vapors of different quantities of liquid mixture: 6 vol.% of studied sorbate + 94 vol.% of H_2O; ▲ and △, $V_S/(P/P_0)_{6\%}$ values of sorption isotherms of 1-propanol and 2-propanol respectively on prehydrated HSA from Table 1.

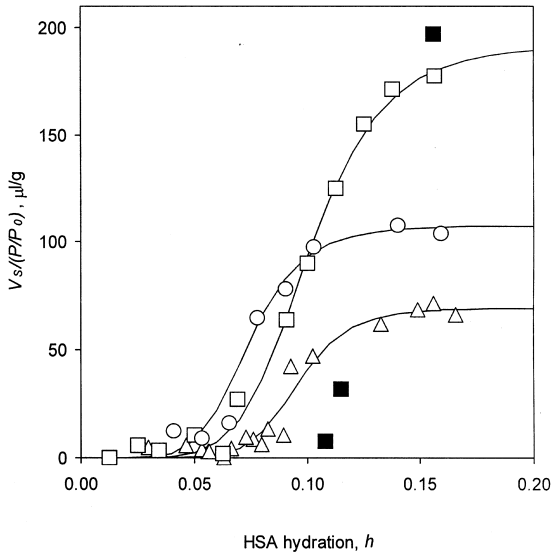

Fig. 5. Sorption isotherms of dioxane (□), ethylacetate (○) and benzene (△) on solid HSA (0.01 h initially) equilibrated at 298 K with vapors of different quantities of liquid mixture: 6 vol.% of organic sorbate + 94 vol.% of H_2O; ■, $V_S/(P/P_0)_{6\%}$ values for sorption isotherms of dioxane on prehydrated HSA from Table 1. Solid lines are the isotherms calculated by Eq. (1).

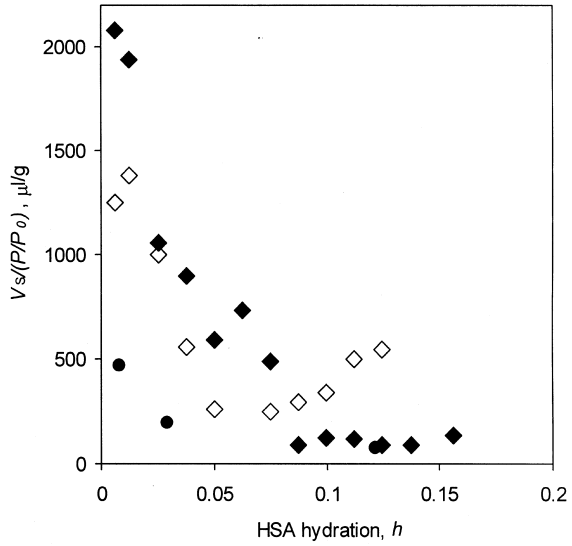

Fig. 6. Sorption isotherms of ethanol (◇) and acetonitrile (◆) on solid HSA (0.01 h initially) equilibrated at 298 K with vapors of different quantities of liquid mixture: 6 vol.% of organic sorbate + 94 vol.% of H_2O; ●, $V_S/(P/P_0)_{6\%}$ values for sorption isotherms of acetonitrile on prehydrated HSA from Table 1.

0.5. The 'active' components (methanol, ethanol, and acetonitrile) do not have significant cooperative influence on ΔH values. The relative order of critical water activities for systems with different organic cosolvents observed for HSA immersion enthalpies [10] is the same as the relative order of critical hydration values obtained in the

Table 2
Parameters of sorption isotherms for initially dry HSA (0.01 h) equilibrated with mixture of 6 vol.% of organic sorbate + 94 vol.% of H_2O in absence of liquid phase at 298 K

N	Sorbate	Critical HSA hydration (h_c) (g H_2O/g HSA)	$[V_S/(P/P_0)]_{sat}$ (µl/g)	$[A/(P/P_0)]_{sat}$ mol sorbate /mol HSA	Log $P_{o/w}$[a]
1	Water[b]	–	219[c]	797	−1.15
2	Dioxane	0.07	192[d]	147	−0.42
3	Acetonitrile	–	94[c]	117	−0.34
4	Ethanol	–	543[c]	608	−0.32
5	2-Propanol	0.06	602[c]	527	0.14
6	1-Propanol	0.04	536[c]	460	0.34
7	Ethylacetate	0.07	108[d]	72	0.83
8	Benzene	0.08	69[d]	51	2.15

[a] Data from ref. [31].
[b] Data from ref. [21].
[c] Data at HSA hydration 0.13 h.
[d] Data calculated by Eq. (1); the standard deviation δ of the approximation (0.03 for dioxane, 0.04 for ethylacetate and 0.05 for benzene) has been estimated for normalized shortest distances between experimental points and calculated line: $\delta = \sqrt{\Sigma\left(((h_{calc} - h_{exp})/h_{max})^2 + (((V_{ads}/(P/P_0))_{calc} - (V_{ads}/(P/P_0))_{exp})/(V_{ads}/(P/P_0))_{max})^2\right)/(n-2)}$.

present work (Table 2). The HSA immersion enthalpy for ternary system with 1-propanol has the lower critical water activity than for systems with dioxane, pyridine, 1-butanol.

In Table 2 the average points of the saturation parts of sorption isotherms $[V_S/(P/P_0)]_{sat}$ are given as $[V_S/(P/P_0)]$ values at 0.13 h for alcohols and acetonitrile. The higher sorption affinity of dried HSA (0.008 h) to 1-propanol than to 2-propanol observed in our previous work [17] can be seen also at HSA hydration up to 0.09 h (Fig. 4). At this hydration point the binding selectivity of HSA to propanols inverses. The ratio of $[V_S/(P/P_0)]_{sat}$ values for 2-propanol and 1-propanol (~ 1.15) still is a bit lower than solution selectivity of liquid water: $\gamma_{1\text{-PrOH}}/\gamma_{2\text{-PrOH}} = 1.76$. The limiting activity coefficients of 1-propanol and 2-propanol in water are equal to $\gamma_{1\text{-PrOH}} = 13.9$ and $\gamma_{2\text{-PrOH}} = 7.9$, respectively [36]. The $[V_S/(P/P_0)]_{sat}$ values for 'passive' substances (benzene, ethylacetate and dioxane) were calculated by approximation of the sorption isotherms (Fig. 5) by the sigmoid function that is commonly used in general form for description of cooperative phenomena in biological systems [37]:

$$V_S/(P/P_0) = [V_S/(P/P_0)]_{sat} Ch^N/(1 + Ch^N) \quad (1)$$

where h is protein hydration in g H_2O/g HSA, N is the cooperativity constant, and C is a binding parameter. Approximation of obtained sorption isotherms for 'passive' substances by shortest normalized distances between experimental points and calculated isotherm gives two stable parameters: $(\ln C)/N$ and $[V_S/(P/P_0)]_{sat}$. The values of $(\ln C)/N$ are equal to 2.35 for benzene, 2.27 for dioxane and 2.60 for ethylacetate. The cooperativity constant N is determined with large error. The calculated values of N are equal to ~ 6 for dioxane and ethylacetate and ~ 9 for benzene. Besides in the Table 2 the values of molar binding affinity of HSA to studied sorbates are presented:

$$[A/(P/P_0)]_{sat} = [V_S/(P/P_0)]_{sat} M_{HSA}/V_M$$

where $M_{HSA} = 66\,000$, the molar weight of HSA, and V_M is the molar volume of sorbate. Comparison between $[A/(P/P_0)]_{sat}$ values for the studied organic sorbates and water and their water/1-octanol partition parameter Log $P_{o/w}$ demonstrates the different behavior of hydroxylic and aprotic sorbates. The studied hydroxylic sorbates have higher binding affinity to hydrated HSA at the same log $P_{o/w}$ values.

Comparison of $V_S/(P/P_0)_{6\%}$ values for prehydrated HSA (Table 1) with $V_S/(P/P_0)$ values on the sorption isotherms for HSA (0.01 h) equilibrated with binary water organic vapor mixture (Figs. 4–6) demonstrates significant history effect. Prehydrated HSA preparation has lower sorption affinity in the most cases and requires the higher critical hydration (Figs. 4 and 5) for effective binding of passive sorbate than HSA (0.01 h) equilibrated with water/organic vapor mixture. The critical HSA hydration value (~ 0.1 h) required for effective vapor sorption of dioxane on prehydrated HSA (Fig. 5) coincides with HSA hydration value at which cooperative water sorption increase and jump of the HSA immersion enthalpy take place for prehydrated HSA (0.1 h) suspended in water + dioxane solvent [7]. Analogous hydration history phenomena have been observed earlier for enzymes. The enzymes equilibrated with water in absence of organic solvent have lower enzymatic activity when suspended in this solvent than the same enzyme preparation hydrated in situ (in the contact with liquid organic solvent) [4,38–40]. Some relation to the observed hydration history effect may be found also in apparent contradiction between results of determination of water contents in protein equilibrated with liquid water/organic mixture by different methods. Determination of protein hydration by the Fischer method shows the increase of the water sorption by chymotrypsinogen suspended in benzene at the fixed water activity a_w above $a_w = 0.5$ compared to water vapor sorption isotherms in absence of organic component [4,6]. The data obtained by ^{2}H-isotope exchange between water bound by subtilisin Carlsberg and excessive quantity of liquid propanol show decrease of water sorption by the protein having the contact with liquid hexane compared to water

sorption in air in absence of hexane at the same water activity [41]. Later the existence of non-exchanging water was observed for the same enzyme suspended in tetrahydrofuran [42].

The ability of alcohols and acetonitrile to serve as media in replacement of water in antigen–antibody binding [5] and ability of alcohols partially to replace the role of water in facilitating catalysis [15] is well known. To examine the effect of the third organic component on the binding properties of solid HSA, we studied the influence of 'active' sorbates (ethanol, acetonitrile) on the sorption of 'passive' sorbates (benzene, dioxane) in the systems of dried HSA (0.01 h) + two organic sorbates. The dilute solutions with 6 vol.% of 'passive' substance in the 'active' solvent were taken as the source of sorbate vapors for each studied system except the case with ethanol + benzene mixture for which the lower concentration of benzene (4 vol.%) was used. We could not obtain reproducible results with 6 vol.% of benzene in ethanol. The sorption isotherms as the plots of $[V_S/(P/P_0)]^{(1)}$ value of 'passive' sorbate vs. sorbed volume $V_S^{(2)}$ of 'active' sorbate are presented in Figs. 7 and 8.

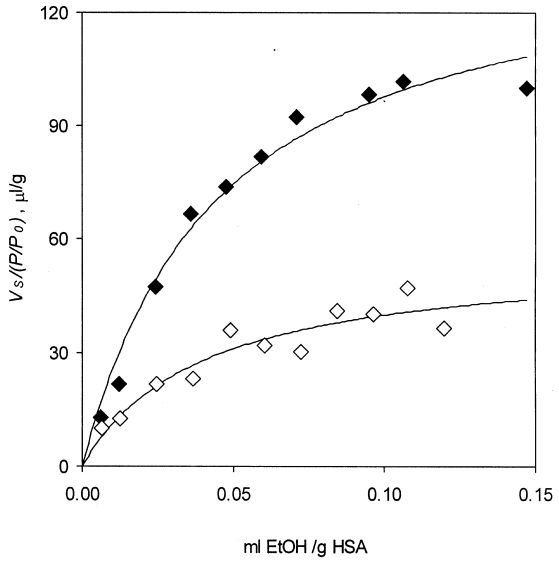

Fig. 7. Sorption isotherms of dioxane (♦), and benzene (◇) on solid HSA (0.01 h initially) equilibrated at 298 K with vapors of different quantities of liquid mixtures: 6 vol.% of dioxane (1) + 94 vol.% of ethanol (2) and 4 vol.% of benzene (1) + 96 vol.% of ethanol (2), respectively. Solid lines are the isotherms calculated by Eq. (2).

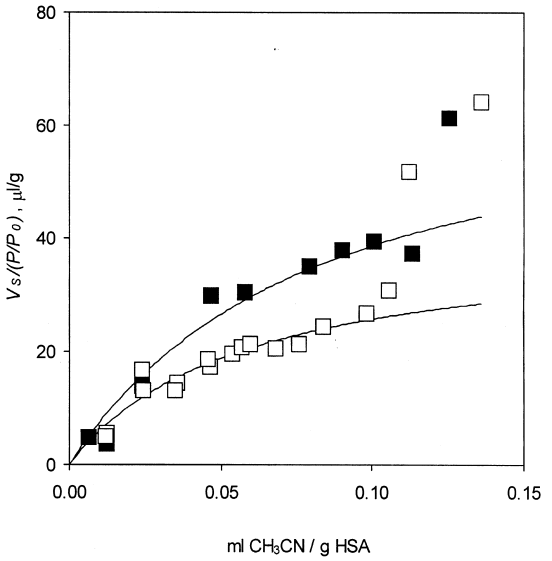

Fig. 8. Sorption isotherms of dioxane (■), and benzene (□) on solid HSA (0.01 h) equilibrated at 298 K with vapors of different quantities of liquid mixture: 6 vol.% of organic sorbate (1) + 94 vol.% of acetonitrile (2). Solid lines are the isotherms calculated by Eq. (2).

The absence of liquid phase in four studied ternary systems with two organic sorbates after equilibration was analyzed by the sum of sorbate activities P/P_0. The analysis was based on the assumption that the binary liquid solution having the positive deviation from the Raoult's law (activity coefficients of both components are greater than 1) exists if the sum of activities of its components is greater than 1. The used binary mixtures of organic sorbates (ethanol + dioxane, ethanol + benzene, acetonitrile + dioxane, acetonitrile + benzene) belong to the group of systems which vapor–liquid equilibrium data can be approximated by the two-parameter Wilson's equation [43]. The last implies monotonous dependence of activity coefficients of components on composition of binary liquid mixture. So to prove the positive deviation from Raoult's law for these binary liquid solutions it is enough to find if for each component the limiting activity coefficient γ^{∞} is greater than 1. The γ^{∞} values determined in the present work and in our earlier works [22,44]

Table 3
Parameters[a] of sorption isotherms for dry HSA (0.01 h) equilibrated with mixture of 6 vol.% of 'passive' sorbate + 94 vol.% of 'active' sorbate in absence of liquid phase and limiting activity coefficients[b] of components in binary liquid mixtures of sorbates at 298 K

'Passive' sorbate (1)	'Active' sorbate (2)	$[V_S/(P/P_0)]_{sat}$ (μl/g)	C'	γ_{12}^{∞}	γ_{21}^{∞}
Dioxane	Ethanol	142	22	3.10 [45]	2.3 [44]; 2.49 [45]
Benzene[c]	Ethanol	56	25	5.2	17.3; 15.18 [45]
Dioxane	Acetonitrile	70	12	1.35; 1.37 [45]	1.24
Benzene	Acetonitrile	40	18	2.9[22]; 3.1[d]	2.65

[a] Calculated by Eq. (2).
[b] γ_{ij}^{∞}-Limiting activity coefficient of solute i in solvent j.
[c] HSA was equilibrated with mixture of 4 vol.% of benzene + 96 vol.% of ethanol.
[d] γ^{∞} value at 298 K calculated by limiting activity coefficient at 293 K from ref. [46] and solution enthalpy from ref. [47].

are given in Table 3. In addition, available data of other authors [45,46] are presented. There is good agreement between our results and literature data. In all studied cases $\gamma^{\infty} > 1$. For studied systems the sum of activities of volatile components (Tables 8A–11A in Appendix A) is below unity except for several points where activity of the major component is above 0.85. In the last case the formation of liquid phase in the system cannot be excluded. It may be the cause of cooperative increase of sorption values V_S of acetonitrile at its activity P/P_0 above 0.85 in the presence of benzene and dioxane (Figs. 9 and 10). The same may be the cause of cooperative increase of HSA sorption affinity value $[V_S/(P/P_0)]^{(1)}$ to benzene and dioxane at high values of sorbed volume $[V_S^{(2)} > 0.10$ ml/g] of acetonitrile on Fig. 8. Three higher points of dioxane in this $V_S^{(2)}$ range were not shown in Fig. 8. The experimental points at activities of major component above 0.85 were not included in the analysis of obtained sorption isotherms in present work.

Unlike the hydration effect the influence of ethanol and acetonitrile on the HSA binding affinity to 'passive' sorbates does not exhibit any threshold by activity of 'active' component. In the presence of ethanol and acetonitrile the increase of $[V_S/(P/P_0)]^{(1)}$ value for 'passive' sorbate (1) take place practically from zero volume $V_S^{(2)}$ of sorbed 'active' sorbate (Figs. 7 and 8). We approximated these isotherms by Eq. (2):

$$[V_S/(P/P_0)]^{(1)} = [V_S/(P/P_0)]_{sat} C' V_S^{(2)} / (1 + C' V_S^{(2)}) \quad (2)$$

where C' is binding parameter, $[V_S/(P/P_0)]_{sat}$ is saturated value of sorption affinity $[V_S/(P/P_0)]^{(1)}$. Eq. (2) is derived from Eq. (1) at $N = 1$ by substitution of HSA hydration h on $V_S^{(2)}$ value in ml/g.

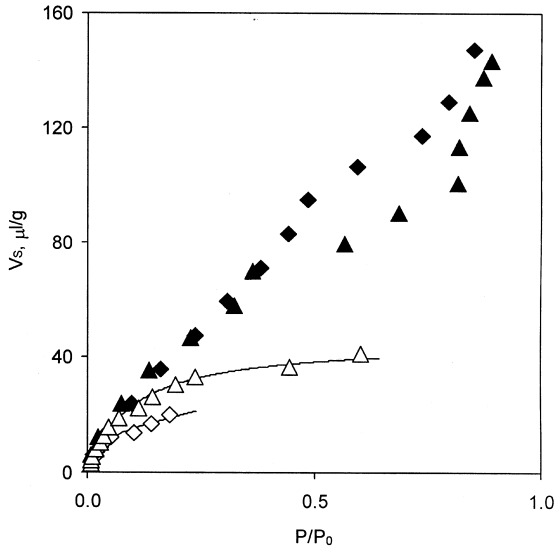

Fig. 9. Sorption isotherms of ethanol (◇♦) and acetonitrile (△▲) on dried HSA at 0.008 h for ethanol (data from [17]) and 0.01 h for acetonitrile (empty points) and on solid HSA (0.01 h) equilibrated at 298 K with vapors of different quantities of liquid mixture: 94 vol.% of the studied sorbate + 6 vol.% of dioxane (filled points). Solid lines are isotherms mentioned in Table 1.

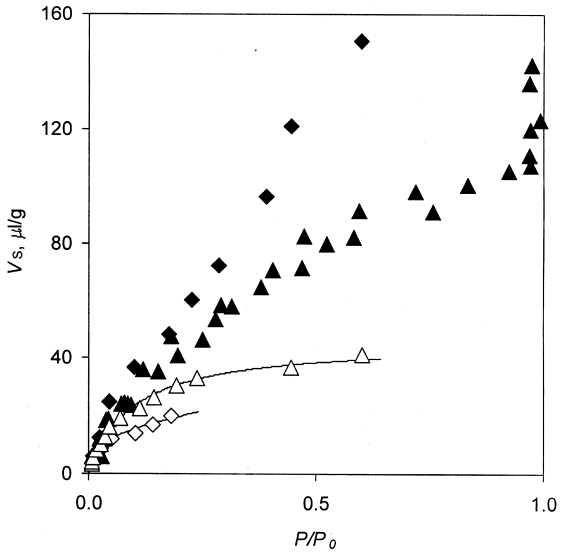

Fig. 10. Sorption isotherms of ethanol (◇ ◆) and acetonitrile (△ ▲) on dried HSA at 0.008 h for ethanol (data from [17]) and 0.01 h for acetonitrile (empty points) and on solid HSA (0.01 h) equilibrated at 298 K with vapors of different quantities of liquid mixtures: 96 vol.% of ethanol + 4 vol.% of benzene and 94 vol.% of acetonitrile + 6 vol.% of benzene (filled points). Solid lines are isotherms mentioned in Table 1.

Calculated values of $[V_S/(P/P_0)]_{sat}$ and C' for studied systems are given in Table 3. The comparison of $[V_S/(P/P_0)]_{sat}$ values obtained for systems with two organic sorbates (Table 3) with data for ternary systems HSA + water + organic sorbate (Table 2) show that ethanol and acetonitrile as 'active' components have lower sorption activation effect on the sorption of 'passive' sorbates than water. This effect is lower for aprotic sorbate acetonitrile than for ethanol. The ratio of $[V_S/(P/P_0)]_{sat}$ values for dioxane and benzene in the ternary systems with water, ethanol and acetonitrile is equal to 2.8, 2.5 and 1.8. This fact demonstrates the reduction of the binding selectivity of HSA in the presence of acetonitrile compared to the systems with water and ethanol. This phenomenon may be related to the solvent effect on substrate specificity of enzymes suspended in organic media [48].

In Figs. 9 and 10 the effect of 'passive' sorbates (benzene, dioxane) on the sorption isotherms of 'active' sorbates (acetonitrile, ethanol) are shown. Obviously the effect of minor 'passive' component on the sorption of major 'active' component is also significant despite the large excess of 'active' components in the studied systems. This effect has the same sign as the cooperative influence of organic component on the sorption of water by HSA suspensions in mixtures of water with dioxane and n-butanol observed on water sorption isotherms above water activity 0.5 or protein hydration 0.10 h [7,9,10]. Analogous is the effect of hydrophobic solvents on the water sorption by suspended in them chymotrypsinogen and alcohol oxidase observed on the sorption isotherms presented as a function of water activity [4,6]. Calculation of the influence of 6 vol.% of 'passive' sorbate on the sorption of 94 vol.% of the active sorbate shows that the sorption of 1 mol of dioxane on the HSA induces additional binding of 9–12 mol ethanol or acetonitrile at the same activity of 'active' sorbate. Approximately the same is the benzene influence on the sorption of studied 'active' sorbates. So this effect also can be regarded as cooperative.

Comparison of the obtained vapor sorption data for dioxane + water mixtures with the data on enthalpies of HSA immersion and water sorption by HSA suspensions in dioxane + water mixtures [7] makes possible the estimation of the enthalpy of dioxane–HSA interaction per 1 mol dioxane. We determined the activity of dioxane $P/P_0 =$ 0.88 in solution of 2.83 M water in dioxane. The corresponding HSA hydration value is 0.17 h. At this solvent composition the immersion enthalpy of prehydrated HSA (0.1 h) reaches the second saturation value after the sharp exothermic increase above the critical HSA hydration level (0.1 h) [7]. The value of this second immersion enthalpy wave obviously induced by dioxane–HSA interaction is approximately −35 J/g. The uptake of dioxane on prehydrated HSA in the present work at the same dioxane activity and HSA hydration is ~120 μl/g or 0.0014 mol/g. So the enthalpy of dioxane binding by prehydrated HSA (0.1 h) suspended in solution of 2.83 M of water in dioxane is equal to $\Delta H \sim$ −25 kJ/mol. This ΔH value is of the same order of magnitude as the binding enthalpies of various substrates for

HSA in water solution being in the interval from -20 to -70 kJ/mol [49]. Probably the large ΔH value for dioxane is due not only to dioxane–HSA molecular interaction but also to cooperative binding of additional water molecules by HSA above the water activity 0.5 observed in Borisover et al. [7].

4. Discussion

Proposed interpretation of observed sorption effects for human serum albumin is closely related to the 'knot-matrix' model used for description of properties of hydrated proteins [13]. According to this model the hydrated globular proteins are assumed to consist of dynamically distinct regions: the 'knots' and 'mobile matrices'. The 'knot' residues can pack very efficiently without compromising the strength of their hydrogen bonding. In 'knots' good packing and strong hydrogen bonding are cooperatively enhanced. In 'matrices' there is a tendency 'either to sacrifice optimum packing for good hydrogen bonding or to pack well at the expense of distorting hydrogen bonds from their optimum geometry' [13]. In terms of this model the molecule of 'passive' sorbate can be the nucleus of the protein 'knot' above the critical protein hydration. As the sorbate enters the 'matrix' the 'knot' forms including neighboring residues and water molecules with cooperative increase of 'good packing' and hydrogen bonding. The result is the observed increase of HSA sorption affinity both to water and 'passive' sorbate in ternary systems as compared to binary systems protein + water and protein + 'passive' sorbate. The last can be hydrophilic or hydrophobic: it probably does not much matter for the binding by this mechanism. But the polar groups in sorbate molecule obviously contribute to binding energy through ordinary pair-wise molecular interactions. The saturated sorption affinity $V_S(P/P_0)_{sat}$ (Table 2) of hydrated HSA to polar dioxane, for example, is higher than to non-polar benzene.

Otherwise the rigid 'knots' that are probably formed by HSA in the studied ternary systems with 'passive' components can be regarded as the 'new entities of higher organization' or supramolecular substrate–receptor complexes in terms of supramolecular chemistry [1]. The complexes of this kind have the 'spatial arrangement of their components, their architecture or superstructure' [1] that correspond to the assumption of 'knot' good packing and optimal hydrogen bonding in the proposed model.

Proposed mechanism of substrate–protein binding implies that the binding of 'passive' sorbate ($\equiv$ substrate) (S) by HSA receptor binding sites (RBS) in studied systems above the critical hydration value is accompanied by binding of $n2$ molecules of water (W) in addition to $n1$ molecules that can be bound in absence of substrate at the same water activity:

$$S + RBS - W_{n1} + n2W \rightleftarrows S \cdot RBS - W_{n1+n2} \quad (3)$$

The same supramolecular mechanism can be proposed for ternary systems with 'active' component other than water because of the synergetic binding of both sorbates observed for HSA in ternary systems with dioxane or benzene as 'passive' component and ethanol or acetonitrile as 'active' component. The analogous model describing the binding of hydrophobic substances by hydrophilic molecule in the presence of water has been proposed for supramolecular complexes of cyclodextrins as one of the five possible hypotheses [50].

In terms of the proposed supramolecular model the receptor properties of the system solid HSA + 'active' component must depend on the ability of the 'active' substance to form the rigid 'knots' within the 'mobile' protein matrix by H-bond bridges. This ability decreases in the row: water > ethanol > acetonitrile, because the molecules of ethanol and especially of acetonitrile cannot form such stable H-bond bridges between aminoacid residues of HSA as water. So we must expect reduction of the HSA sorption affinity properties to 'passive' sorbates (benzene, dioxane) in the same row of 'active' components despite approximately the same binding affinity of the dried HSA

(0.008 h) to ethanol and acetonitrile [17]. The observed order of 'active' component effect on the saturated sorption affinity $[V_S/(P/P_0)]_{sat}$ of HSA to 'passive' sorbates (Tables 2 and 3, Figs. 7 and 8) corresponds to this proposal.

The much weaker or no ability of ethanol and acetonitrile to form H-bond bridges between HSA aminoacid residues is probably the cause of absence of threshold or critical activity of these 'active' substances necessary for the sorption activation of the studied 'passive' sorbates as distinct from the hydration effect. Coordination of each 'active' molecule with only one residue may produce more 'mobile' protein matrix compared to coordination with formation of H-bond bridges. For hydrated protein the 'mobile' matrix is formed probably only after all possible monomolecular water bridges are formed in the HSA molecules. If this situation corresponds to the participation of each aminoacid residue of the HSA molecule in only one bridge with other residue, the threshold value of HSA hydration must be 0.08 h. This value is approximately equal to the critical hydration level for studied ternary systems with dioxane, ethylacetate and benzene (Table 2). The lower critical HSA hydration level for sorption of propanols (Table 2) may be stipulated by their bifunctional behavior: as the 'active' components participating in formation of 'mobile' protein matrix and as substrates.

Supposed formation of rigid 'knots' or supramolecular complexes may lead to very slow equilibration rate in studied ternary systems HSA + water + organic component and to observed hydration history effect as well as buried water is assumed to be a source of sorption–desorption hysteresis for hydrated proteins [13]. If formed the rigid supramolecular entities can contribute to microheterogeneous structure of hydrated protein supposed earlier [13,51] and to the trapping of initial protein conformation. The last has been assumed to be a cause of hydration history phenomenon for hydrated enzymes treated by anhydrous organic solvents [40], and for enzymes suspended in water/organic mixtures by different methods [38]. The changes in microscopic structure of hydrated enzyme powder due to its exposition to organic solvent were referred to explain the divergence in rates observed between the different equilibration methods [39].

Acknowledgements

The authors gratefully acknowledge Vladimir A. Sirotkin (Kazan State University) and Mikhail D. Borisover (Institute of Soils and Water, The Volcani Center, Bet Dagan, Israel) for helpful discussion.

Appendix A:

Data on sorption isotherms for ternary systems prepared by vapor sorption of different amounts of liquid mixture of X vol.% of sorbate (1) + (100 $-X$) vol.% of sorbate (2) added to initially dry HSA (0.01 ± 0.003 h) in hermetically closed vials at 298 K

Table A1

6 vol.% n-PrOH(1) + 94 vol.% water (2)			Binary system HSA + n-PrOH
Final HSA hydration (h)	$(P/P_0)^{(1)}$	$V_S^{(1)}$ (μl/g)	V_S* (μl/g)
0.006	0.0026	0.39	0.36
0.013	0.0071	0.77	0.94
0.025	0.011	1.55	1.42
0.041	0.016	2.52	1.96
0.056	0.014	3.53	1.72
0.069	0.012	4.34	1.52
0.075	0.011	4.74	1.44
0.081	0.011	5.15	1.42
0.087	0.010	5.55	1.30
0.10	0.010	6.35	1.33
0.11	0.012	7.14	1.54
0.12	0.014	7.93	1.75
0.14	0.017	8.72	2.07
0.16	0.021	9.90	2.44

* Data are calculated on the basis of BET equation with parameters obtained for binary system n-PrOH + dried HSA (0.008 ± 0.002 h) from Table 1.

Table A2
6 vol.% i-PrOH (1) + 94 vol.% water (2)

Final HSA hydration (h)	$(P/P_0)^{(1)}$	$V_S^{(1)}$ (μl/g)
0.006	0.0054	0.34
0.013	0.013	0.67
0.025	0.017	1.43
0.041	0.023	2.36
0.056	0.032	3.27
0.069	0.019	4.20
0.075	0.018	4.61
0.081	0.014	5.05
0.087	0.011	5.49
0.10	0.0086	6.31
0.11	0.012	7.07
0.12	0.013	7.87
0.14	0.015	8.64
0.16	0.018	9.82

Table A3
6 vol.% Dioxane (1) + 94 vol.% water (2)

Final HSA hydration (h)	$(P/P_0)^{(1)}$	$V_S^{(1)}$ (μl/g)
0.025	0.079	0.083
0.038	0.11	0.63
0.047	0.18	0.58
0.063	0.16	1.71
0.075	0.36	0.74
0.08	0.122	3.3
0.10	0.079	5.1
0.11	0.065	5.8
0.13	0.054	6.7
0.14	0.049	7.6
0.15	0.049	8.4
0.17	0.054	9.5

Table A4
6 vol.% EtOAc (1) + 94 vol.% water (2)

Final HSA hydration (h)	$(P/P_0)^{(1)}$	$V_S^{(1)}$ (μl/g)
0.047	0.059	0.74
0.060	0.086	0.78
0.072	0.090	1.47
0.085	0.052	3.35
0.097	0.053	4.12
0.11	0.051	4.97
0.15	0.065	6.98
0.17	0.076	7.87

Table A5
6 vol.% Benzene (1) + 94 vol.% water (2)

Final HSA hydration (h)	$(P/P_0)^{(1)}$	$V_S^{(1)}$ (μl/g)
0.037	0.062	0.29
0.057	0.12	0.50
0.061	0.14	0.40
0.063	0.13	0.38
0.077	0.14	1.4
0.073	0.15	0.65
0.083	0.15	1.3
0.084	0.17	1.1
0.090	0.14	1.8
0.094	0.17	1.8
0.099	0.087	3.7
0.11	0.090	4.3
0.14	0.098	6.1
0.16	0.10	7.0
0.16	0.11	7.6
0.17	0.12	7.9

Table A6

6 vol.% EtOH (1) + 94 vol.% water (2)			Binary system HSA + EtOH V_S* (μl/g)
Final HSA hydration (h)	$(P/P_0)^{(1)}$	$V_S^{(1)}$ (μl/g)	
0.016	0.0003	0.40	0.17
0.023	0.0006	0.79	0.31
0.035	0.0016	1.58	0.84
0.047	0.004	2.35	2.08
0.060	0.0117	3.07	4.86
0.085	0.018	4.60	6.69
0.097	0.018	5.40	6.66
0.11	0.018	6.20	6.63
0.12	0.014	7.04	5.56
0.13	0.014	7.84	5.66
0.15	0.076	7.96	13.9
0.17	0.059	9.35	12.5

* Data are calculated by BET equation with parameters ($V_m = 17.7$, $C = 31.3$) obtained for binary system EtOH + dried HSA (0.008 ± 0.002 h) [17].

Table A7

6 vol.% CH_3CN (1) + 94 vol.% water (2)			Binary system HSA + CH_3CN
Final HSA hydration (h)	$(P/P_0)^{(1)}$	$V_S^{(1)}$ (μl/g)	V_S* (μl/g)
0.016	0.0002	0.40	0.20
0.023	0.00041	0.79	0.42
0.035	0.0015	1.58	1.44
0.047	0.0026	2.36	2.42
0.060	0.0053	3.12	4.35
0.072	0.0054	3.92	4.41
0.085	0.0095	4.66	6.76
0.10	0.052	4.83	15.9
0.11	0.046	5.72	15.2
0.12	0.053	6.41	16.0
0.13	0.075	6.89	17.8
0.15	0.082	7.58	18.3
0.17	0.065	9.04	17.1

*Data are calculated by Langmure equation with parameters obtained for binary system CH_3CN + dried HSA (0.01 h) from Table 1.

Table A8
6 vol.% Dioxane (1) + 94 vol.% EtOH (2)

$P/P_0^{(2)}$	$(P/P_0)^{(1)}$	$V_S^{(2)}$ (μl/g)	$V_S^{(1)}$ (μl/g)
0.013	0.017	6.1	0.22
0.034	0.025	12.2	0.54
0.096	0.028	24.0	1.32
0.16	0.031	35.8	2.08
0.24	0.038	47.5	2.81
0.31	0.044	59.3	3.56
0.44	0.055	82.9	5.05
0.48	0.059	94.9	5.80
0.59	0.064	106.3	6.55
0.85	0.091	147.3	9.08

Table A9
4 vol.% Benzene (1) + 96 vol.% EtOH (2)

$P/P_0^{(2)}$	$(P/P_0)^{(1)}$	$V_S^{(2)}$ (μl/g)	$V_S^{(1)}$ (μl/g)
0.01	0.007	6.3	0.07
0.03	0.014	12.5	0.17
0.09	0.023	24.6	0.49
0.17	0.033	36.6	0.76
0.22	0.035	48.8	1.24
0.33	0.046	60.4	1.48
0.42	0.057	72.2	1.73
0.48	0.056	84.3	2.30
0.55	0.065	96.4	2.61
0.67	0.066	107.9	3.11
0.74	0.086	119.9	3.13

Table A10
6 vol.% Dioxane (1) + 94 vol.% CH_3CN (2)

$P/P_0^{(2)}$	$(P/P_0)^{(1)}$	$V_S^{(2)}$ (μl/g)	$V_S^{(1)}$ (μl/g)
0.008	0.027	6.1	0.13
0.023	0.058	12.2	0.21
0.074	0.066	24.0	0.93
0.23	0.080	46.8	2.4
0.32	0.099	57.9	3.0
0.57	0.12	79.4	4.3
0.69	0.13	90.1	5.0
0.82	0.15	100.7	5.7
0.82	0.17	113.2	6.3
0.84	0.12	125.4	7.6
0.87	0.069	137.5	8.9
0.89	0.070	143.5	9.3

Table A11
6 vol.% Benzene (1) + 94 vol.% CH_3CN (2)

$P/P_0^{(2)}$	$(P/P_0)^{(1)}$	$V_S^{(2)}$ (μl/g)	$V_S^{(1)}$ (μl/g)
0.025	0.026	12.2	0.14
0.032	0.026	12.1	0.13
0.071	0.041	24.0	0.54
0.092	0.038	23.7	0.63
0.15	0.060	35.3	0.87
0.20	0.062	34.7	0.81
0.25	0.074	46.4	1.3
0.28	0.053	53.5	1.0
0.31	0.072	45.6	1.3
0.40	0.086	56.7	1.8
0.43	0.089	59.5	1.9
0.48	0.10	68.1	2.1
0.60	0.11	75.8	2.5
0.69	0.12	83.8	2.9
0.72	0.086	98.2	2.3
0.90	0.10	112.1	5.3
0.92	0.13	105.4	4.1
0.97	0.11	136.1	6.9

References

[1] J.-M. Lehn, Supramolecular Chemistry, VCH, Weinheim, 1995.
[2] A.M. Klibanov, Trends Biotech. 15 (1997) 97.
[3] G. Bell, P.J. Halling, B.D. Moore, J. Partridge, D.G. Rees, Trends Biotech. 13 (1995) 468.
[4] P.J. Halling, Enzyme Microb. Technol. 16 (1994) 178.
[5] A.J. Russell, L.J. Trudel, P.L. Skipper, J.D. Groopman, S.R. Tannenbaum, A.M. Klibanov, Biochem. Biophys. Res. Commun. 158 (1989) 80.
[6] P.J. Halling, Biochim. Biophys. Acta 1040 (1990) 225.

[7] M.D. Borisover, V.A. Sirotkin, B.N. Solomonov, J. Phys. Org. Chem. 8 (1995) 84.
[8] M.D. Borisover, V.A. Sirotkin, B.N. Solomonov, Thermochim. Acta 254 (1995) 47.
[9] M.D. Borisover, V.A. Sirotkin, B.N. Solomonov, Thermochim. Acta 284 (1996) 263.
[10] V.A. Sirotkin, M.D. Borisover, B.N. Solomonov, Biophys. Chem. 69 (1997) 239.
[11] K. Griebenow, A.M. Klibanov, Biotechnol. Bioeng. 53 (1997) 351.
[12] K. Griebenow, A.M. Klibanov, J. Am. Chem. Soc. 118 (1996) 11695.
[13] R.B. Gregory, in: R.B. Gregory (Ed.), Protein–Solvent Interactions, Vol. 264, Marcel Dekker, New York, 1995, p. 191.
[14] O. Almarsson, A.M. Klibanov, Biotechnol. Bioeng. 49 (1996) 87.
[15] G. Bell, A.E.M. Janssen, P.J. Halling, Enzyme Microb. Technol. 20 (1997) 471.
[16] E. Barzana, A.M. Klibanov, M. Karel, Appl. Biochem. Biotechnol. 15 (1987) 25.
[17] V.V. Gorbatchuk, M.A. Ziganshin, B.N. Solomonov, M.D. Borisover, J. Phys. Org. Chem. 10 (1997) 901.
[18] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153.
[19] M. Keits, Technique of Lipidology [in Russian], Mir, Moscow, 1995.
[20] D.D. Perrin, W.L.F. Armarego, D.R. Perrin, Purification of Laboratory Chemicals, Pergamon Press, Oxford, 1980.
[21] H.B. Bull, J. Am. Chem. Soc. 66 (1944) 1499.
[22] V.V. Gorbatchuk, S.A. Smirnov, B.N. Solomonov, A.I. Konovalov, Zh. Obsch. Khim. 60 (1990) 1200.
[23] B. Kolb, P. Pospisil, T. Borath, M. Auer, J. High Res. Chromatogr. Chromatogr. Commun. 2 (1979) 283.
[24] T. Boublik, E. Hala, V. Fried, The Vapour Pressures of Pure Substances, Elsevier, Amsterdam, 1973.
[25] M. Lencka, A. Szafranski, A. Maczynski, Verified Vapor Pressure Data, Vol. 1, PWN, Warszawa, 1984.
[26] A. Hussam, P.W. Carr, Anal. Chem. 57 (1985) 793.
[27] S. Brunauer, P.H. Emmett, E. Teller, J. Am. Chem. Soc. 60 (1938) 309.
[28] V.V. Gorbatchuk, B.N. Solomonov, Zh. Fiz. Khim. (Russ) 70 (1996) 723.
[29] B. Xing, W.B. McGill, M.J. Dudas, Can. J. Soil Sci. 74 (1994) 465.
[30] F. Yang, A.J. Russell, Biotechnol. Bioeng. 49 (1996) 709.
[31] A. Leo, C. Hansch, D. Elkins, Chem. Rev. 71 (1971) 525.
[32] E.M. Ostolovsky, A.D. Botsjansky, S.N. Boriseenko, N.V. Tolkacheva, Biopolymery. Kletka 6 (1990) 59.
[33] Yu.I. Khurgin, E.Yu. Maksareva, Bioorg. Khim. 17 (1991) 76.
[34] V.Ya. Roslyakov, Yu.I. Khurgin, Biokhimiia 37 (1972) 493.
[35] A. Zaks, A.M. Klibanov, J. Biol. Chem. 263 (1988) 8017.
[36] M.H. Abraham, G.S. Whiting, R. Fuchs, E.J. Chambers, J. Chem. Soc. Perkin Trans. 2 (1990) 291.
[37] M. Abundo, L. Accardi, A.F. Agro, G. Mei, N. Rosato, Biophys. Chem. 58 (1996) 313.
[38] T. Ke, A.M. Klibanov, Biotechnol. Bioeng. 57 (1998) 746.
[39] M.C. Parker, B.D. Moore, A.J. Blacker, Biocatalysis 10 (1994) 269.
[40] J. Partridge, G.A. Hatcheon, B.D. Moore, P.J. Halling, J. Am. Chem. Soc. 118 (1996) 12873.
[41] M.C. Parker, B.D. Moore, A.J. Blacker, Biotechnol. Bioeng. 46 (1995) 452.
[42] C.S. Lee, M.T. Ru, M. Haake, J.S. Dordick, J.A. Reimer, D.S. Clark, Biotechnol. Bioeng. 57 (1998) 686.
[43] M. Hirata, S. Ohe, K. Nagahama, Computer Aided Data Book of Vapor–Liquid Equilibria, Kodansha Ltd, Tokyo, Elsevier, New York, 1975.
[44] V.V. Gorbatchuk, S.A. Smirnov, B.N. Solomonov, A.I. Konovalov, Zh. Obsch. Khim. 60 (1990) 1441.
[45] J.H. Park, A. Hussam, P. Couasnon, D. Fritz, P.W. Carr, Anal. Chem. 59 (1987) 1970.
[46] E.R. Thomas, B.A. Newman, T.C. Long, D.A. Wood, C.A. Eckert, J. Chem. Eng. Data. 27 (1982) 399.
[47] B.N. Solomonov, A.I. Konovalov, V.B. Novikov, A.N. Vedernikov, M.D. Borisover, V.V. Gorbatchuk, I.S. Antipin, Zh. Obsch. Khim. 54 (1984) 1622.
[48] C.A. Wescott, A.M. Klibanov, Biotechnol. Bioeng. 56 (1997) 340.
[49] N. Aki, M. Goto, M. Yamamoto, Thermochim. Acta 251 (1995) 379.
[50] K.A. Connors, Chem. Rev. 97 (1997) 1325.
[51] J.L. Green, J. Fan, C.A. Angell, J. Phys. Chem. 98 (1994) 13780.