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ISOTHERM OF WATER SORPTION BY HUMAN SERUM ALBUMIN IN DIOXANE: COMPARISON WITH CALORIMETRIC DATA

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The dependence of the amount of water bound to human serum albumin (HSA) suspended in water–dioxane mixtures vs the equilibrium water concentration in the liquid phase was determined by the Fisher method at 298 K. The Langmuir model was used in order to describe the isotherm of the sorption of water by HSA at low water concentrations in the solvent. The calculated equilibrium constant of water adsorption (3.8 ± 0.6 l mol^{-1}) is in good agreement with the adsorption constant obtained earlier from calorimetric data. The comparison of the determined isotherm of water sorption by HSA with the reported enthalpies of suspension formation showed that at low water concentrations in the solvent, water sorption is the only process contributing to the heat effects of the formation of the 'protein + liquid' heterogeneous system. From this comparison, the enthalpy of water adsorption by HSA was evaluated as -11.9 ± 1.7 kJ mol^{-1}. At higher water concentrations in the solvent the amount of water adsorbed by HSA increased considerably. This increase in the amount of water on HSA at water activities above 0.5 is assumed to be due to the enlargement of the protein surface area.

INTRODUCTION

Enzymes suspended in nearly anhydrous solvents can act as catalysts.[1–3] This ability appears to hold considerable promise for chemical technology and basic research. However, the potential of protein functioning depends essentially on the extent of hydration of the solid catalyst.[3–9] Therefore information on the thermodynamics of the binding of water by solid proteins in organic solvents is of importance in explaining various protein activities. There are a number of reports on the sorption of water from organic solvents and from the gas phase by proteins.[5,6,8,10–13]

Earlier we proposed a calorimetric approach to the investigation of intermolecular (and/or intramolecular) interactions that occur on placing proteins in organic liquids and water–organic mixtures.[14–16] This approach involved the measurement of the enthalpies corresponding to the formation of the 'protein + liquid' heterogeneous system. The starting point for the measured enthalpies was the partially hydrated protein plus water–organic mixtures. Such an approach was also used to estimate the thermodynamic parameters of water adsorption by human serum albumin (HSA) suspended in water–organic mixtures.[15,16]

In the present work we performed the direct determination of the isotherm of water sorption by HSA in the same water–dioxane mixtures that were previously studied calorimetrically.[15] We intended to obtain support for the validity of the calorimetric estimation of the thermodynamic parameters of water adsorption by proteins suspended in organic solvents. The second aim was to establish whether water sorption is the only process contributing to the heat effects corresponding to the formation of the 'protein + liquid' heterogeneous system.

EXPERIMENTAL

Human serum albumin (HSA) was obtained from Sigma (product No. A1887). 1,4-Dioxane was purified and dried by refluxing over sodium according to the recommended method.[17] Water used for preparation of water–dioxane mixtures was doubly distilled.

The amount of water bound to the suspended protein and the equilibrium water content in the solvent were determined by the Fisher method according to the recommendations.[18] In general, the technique of determination was similar to that described earlier.[5] A 5 mg amount of HSA and 4 ml of water–dioxane mixture were placed in a preweighed glass ampoule, which was closed with a preweighed stopper. After the ampoule had been thermostated for 2 h at 25 °C, an aliquot of the solvent was removed from the ampoule with syringe. Measurement of the equilibrium water content in the aliquot of the solvent was immediately performed

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electrochemically in the Fischer reagent medium. Such extraction of the aliquots and the electrochemical measurement were repeated three times for 40–60 min. Reproducible values of the equilibrium water content in the solvent were obtained. Then the bulk of the liquid phase was withdrawn from the ampoule with a syringe and the ampoule was weighted again. The apparent weight of the remaining liquid phase was calculated as the difference between the final weight of the sealed ampoule and the sum of the weights of the empty ampoule, the stopper and the dry protein. To measure the amount of water on the suspended HSA, the ampoule containing the HSA sample and a small amount of the remaining liquid was transferred into the Fischer apparatus.

After the bottom or the ampoule had been broken off, the total amount of water on HSA (and in the remaining liquid) was measured. Some part of the solvent could have been absorbed by the stopper. To take this into account, the stopper was weighed after the experiment. To determine the true weight of the remaining liquid phase the difference in the weight of the stopper was subtracted from the apparent weight of the remaining solvent. In most cases the ratio of the weight of the remaining liquid phase to the weight of the solid protein was 10–15.

The amount of water in the remaining liquid was evaluated using the weight of the remaining liquid phase, its water content measured previously and the liquid density. The amount of water on HSA corresponds to the difference between the total measured amount of water in the ampoule and the amount of water in the remaining liquid phase. This amount of water on HSA was expressed as percentage by weight with respect to the dry protein (%, w/w).

The amount of water bound to the protein did not depend on the exposure time of HSA in water–dioxane mixtures. This was confirmed by water sorption measurements at equilibrium water concentrations 0·02, 0·8 and 1·37 mol l^{-1}. No noticeable variation of the amount of water on HSA was observed during 20 h.

HSA was insoluble in all the water–dioxane mixtures studied, as found previously.[15]

The initial HSA sample contained 10·1% (w/w) of water (weight with respect to the veight of dry HSA). This was found according to the above-mentioned Fischer method. This water content of HSA corresponds to a value of 10·2% (w/w) measured on a Setaram microthermoanalyser based on the weight loss of the protein sample at 298 K and 3×10^{-3} Torr (1 Torr = 133·3 Pa).

Calculations of the thermodynamic activities for water used in the discussion of the experimental data were made according to equation $a_w = \gamma_w x_w$, where x_w is the mole fraction of water and γ_w is the mole fraction based activity coefficient for water. The mole fraction of water was calculated from the water concentration. To estimate the γ_w values, the literature data[19] for vapour–liquid equilibrium in water–dioxane mistures were approximated by the equation $\ln \gamma_w = a_1 + a_2(1 - x_w)^2$ (at $a_1 = -0{\cdot}059 \pm 0{\cdot}077$, $a_2 = 1{\cdot}904 \pm 0{\cdot}157$ and the standard deviation $s_0 = 0{\cdot}06$).

RESULTS AND DISCUSSION

The amount of water bound to the suspended HSA is plotted against the equilibrium water concentration C_w in water–dioxane mixtures in Figure 1. The dependence obtained can be subdivided into two regions. At the beginning the amount of water on HSA increases with increase in the equilibrium water concentration in the solvent and reaches saturation (C_w range = 0·02–1·5 mol l^{-1}). Further, on increasing the water content in the solvent, the amount of water bound to HSA increases markedly once again. Hence the discussion of the experimental data will be subdivided into two corresponding subsections.

Region of C_w values below 1·5 mol l^{-1}

The dependence of the formation enthalpies of 'HSA + water + dioxane' suspensions on the low water content in the liquid phase was shown[15] to obey the Langnuir model of water adsorption by the protein. From this model, the adsorption equilibrium constant and the energy of monolayer formation were estimated. Therefore, to describe the beginning of the dependence in Figure 1 we also applied the Langmuir model in the

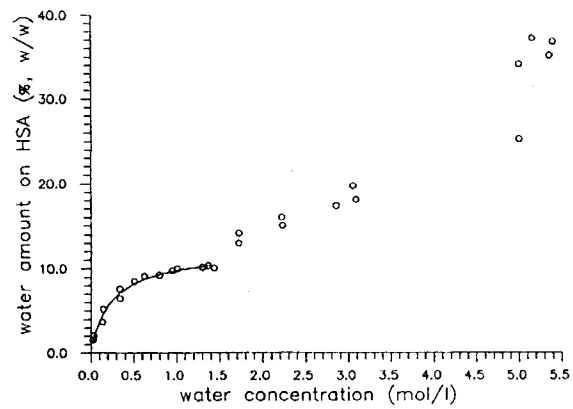

Figure 1. Amount of water on HSA plotted vs the equilibrium water concentration C_w in water–dioxane mixtures at 298 K. The C_w range is 0·021–5·4 mol l^{-1}. The solid line was fitted according to the model equation [number of fitted points $n = 16$, residual standard deviation $s_0 = 0{\cdot}37\%$ (w/w)]

following form:

$$A = A_0 \frac{K_c^{ads} C_w}{1 + K_c^{ads} C_w} + A'$$

where A is the amount of water bound to HSA (%, w/w) and K_c^{ads} is the adsorption constant ($l\ mol^{-1}$) corresponding to the Langmuir equilibrium 'sorption site + water $\rightleftharpoons$ sorption complex.' It should be borne in mind that the suspended protein may have a non-equilibrium structure. Hence the adsorption constant should be considered as a certain effective value describing the water binding by the suspended protein. C_w is the equilibrium concentration of water in the solvent ($mol\ l^{-1}$). A_0, in accordance with the model, is the amount of water in the filled monolayer (%, w/w). A' was introduced into the equation to test the experimental data for a zero offset.

The experimental data in Figure 1 were fitted within the framework of the above equation by the non-linear regression procedure in the C_w range 0–1·5 $mol\ l^{-1}$.

The adsorption equilibrium constant $K_c^{ads} = 3{\cdot}8 \pm 0{\cdot}6\ l\ mol^{-1}$, $A_0 = 11{\cdot}7 \pm 0{\cdot}4\%$ (w/w) (or $6{\cdot}5 \times 10^{-3}\ mol\ g^{-1}$) and $A' = 0{\cdot}5 \pm 0{\cdot}3\%$ (w/w) were estimated from the model. Using these estimated K_c^{ads}, A_0 and A' values, we also calculated the dependence of the amount of water bound to HSA on the C_w value. The calculated dependence is depicted as the solid line in Figure 1. From Figure 1 one can see that the simple Langmuir model represents the experimental data well enough at low water contents, in the liquid phase.

The A' value does not differ essentially from zero. This means that almost all water bound to the initial HSA preparation takes part in the adsorption equilibrium characterized by a single equilibrium constant.

The K_c^{ads} value is close to the adsorption equilibrium constant $K_c^{cal} = 2{\cdot}3 \pm 0{\cdot}7\ l\ mol^{-1}$ evaluated earlier[15] from the calorimetric data on the formation enthalpies of 'HSA + water + dioxane' suspensions. This result lends credence to the view that both the described technique of sorption determination and the calorimetric measurement of suspension formation enthalpies may be applied in an effort to estimate the thermodynamic parameters of the water adsorption by proteins.

Now, to approach the second problem posed in the Introduction, let us correlate directly the enthalpies (or heat effects) of suspension formation with the change in the amount of water on HSA on suspending the protein sample. This correlation is presented in Figure 2.

One can see in Figure 2 that the enthalpies of suspension formation depend linearly on the change in the amount of water on HSA. The intercept of this dependence is close to zero. This result and the agreement between the adsorption equilibrium constants obtained directly and calorimetrically confirm the view that the sorption of water by HSA solely contributes to the heat

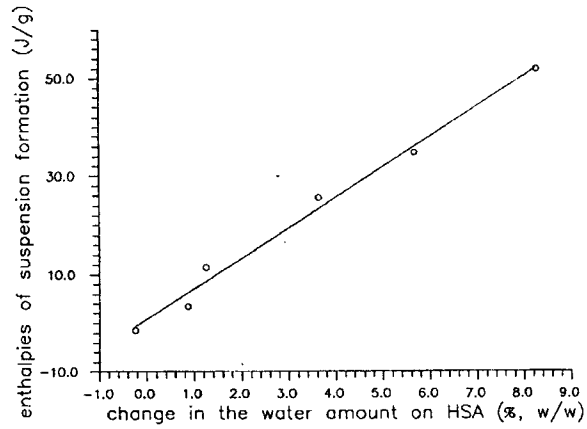

Figure 2. Correlation between the enthalpies of suspension formation (averaged data from Ref. 15, in joules per gram of dry HSA) and the changes in the amount of water on HSA on introducing the HSA sample into water–dioxane mixtures. The change in the amount of water bound to HSA was calculated as the difference between the amount of water on the initial HSA preparation (10·1% (w/w)] and the water amount on the suspended HSA. The C_w range is 0·02–1·5 $mol\ l^{-1}$. The slope of the linear dependence is $6{\cdot}19 \pm 0{\cdot}33$, the intercept is $0{\cdot}81 \pm 1{\cdot}47$, the correlation coefficient $r = 0{\cdot}994$ and the residual standard deviation $s_0 = 2{\cdot}4$.

effects of the 'HSA + liquid' suspension formation at low water contents in water–dioxane mixtures.

Let us now consider in greater detail the thermodynamics of the adsorption equilibrium on the protein. The slope of the line in Figure 2 corresponds to the value opposite in sign to the adsorption enthalpy expressed in joules per gram of water. Consequently, the adsorption enthalpy Δh is $-619 \pm 33\ J\ g^{-1}$. This Δh value may be more correctly calculated in another way. It is known[15] that the energy of water monolayer formation on HSA in water–dioxane mixtures, $A_0 \Delta h$, estimated within the framework of the Langmuir model is $-77{\cdot}4 \pm 8{\cdot}4\ J\ g^{-1}$. The division of the $A_0 \Delta h$ value by A_0 results in the enthalpy Δh of the Langmuir adsorption equilibrium which was found to be $-662 \pm 95\ J\ g^{-1}$ (or $-11{\cdot}9 \pm 1{\cdot}7\ kJ\ mol^{-1}$) and does not differ from the value calculated above. Hence the thermodynamics of the adsorption of water molecules by HSA in water–dioxane mixtures can be summarized at 298 K as follows: the Gibbs free energy $\Delta G = -3{\cdot}3 \pm 0{\cdot}4\ kJ\ mol^{-1}$ ($\Delta G = -RT \ln K_c^{ads}$) and the enthalpy of the water adsorption equilibrium $\Delta h = -11{\cdot}9 \pm 1{\cdot}7\ kJ\ mol^{-1}$.

Region of C_w values above 1·5 mol l^{-1}

To discuss the considerable increase in the amount of water on HSA at water concentrations above 1·5 $mol\ l^{-1}$

in Figure 1, the C_w values were converted into the values of the thermodynamic activity of water, a_w, as described under Experimental.

The amount of water on HSA is presented as a function of the water activity in water–dioxane mixtures in Figure 3. The data on the enthalpies of the suspension formation are also shown in Figure 3. Both the sorption data and calorimetric values in Figure 3 demonstrate that the state of the protein changes substantially in a relatively narrow range of the water activity (0·5–0·73).

The substantial increase in the amount of water on HSA at high water activities corresponds to literature data on the sorption of water by proteins from the gas phase[10–13] and from some water-immiscible organic solvents[10] (e.g. benzene, *tert*-amyl alcohol, ethyl acetate). Such an increase in the water uptake is usually interpreted in terms of multilayer adsorption.[11] However, it should be borne in mind that dioxane is a hydrophilic solvent that is miscible with water in all proportions. Therefore it is difficult to consider the significant increase in the amount of water on HSA as only the result of multilayer adsorption. Hence we venture the hypothesis that at a certain extent of hydration the structure of the previously lyophilized HSA can change relatively abruptly. This change in the protein structure is followed by an increase in the protein surface area, an increase in the amount of water on HSA and negative heat effects. In this case, the enthalpies of suspension formation do not differ essentially from the solution enthalpy of HSA in water ($-43·5 \pm 3·8$ J g^{-1}), as discussed earlier.[15,16]

Interestingly, earlier it was demonstrated[5] by the acylation of free amino groups of mushroom polyphenol oxidase in hexyl acetate that the number of titratable amino groups increases sharply when the water content in the solvent is raised. This increase was interpreted[5] in terms of decreased rigidity of the polyphenol oxidase and can be considered also as an increase in the accessible protein surface area. Moreover, at approximately 20% water on the enzyme, the extent of the acylation of free amino groups of mushroom polyphenol oxidase corresponded to that in pure water. This was considered to support the previously reported finding[20] that the molecular motions of lysozyme containing 15% water are the same as those in aqueous solution. The surprising aspect is that the close agreement between the enthalpy of suspension formation and the solution enthalpy of HSA in water also occurs at approximately 20% water on HSA. Apparently, the protein returns to its original state in the aqueous solution (or close to it). It seems reasonable to assume that such a process can contribute to the formation enthalpies of the 'HSA + liquid' suspensions at high water contents in the solvent.

We conclude that the combination of data on water sorption with the calorimetric enthalpies of suspension formation provides a useful tool for estimating interactions that occur on placing proteins in organic liquids.

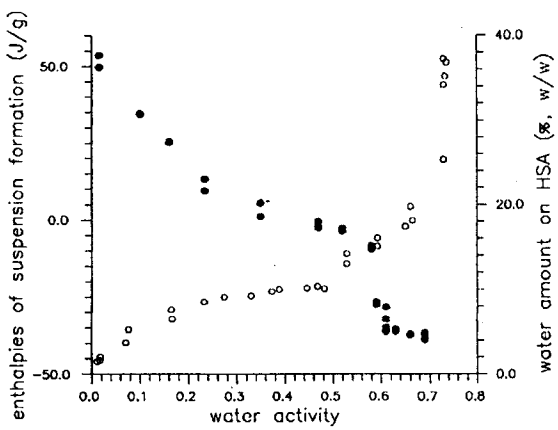

Figure 3. Amount of water on HSA (○) and enthalpies of suspension formation (●) (in joules per gram of dry HSA, from Ref. 15) plotted vs water activity a_w in water–dioxane mixtures at 298 K. The a_w range is 0·01–0·73

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