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ORIGINAL ARTICLE

The environment controlled effect of thiacalix[4]arene on the transition thermodynamics and kinetics of bovine serum albumin

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Abstract Complexation ability of water-soluble thiacalix[4]arene-tetrasulfonate towards three aromatic amino acids (Phenylalanine, Tyrosine and Tryptophane) was studied in water–ethanol mixtures by photoluminescence (PL) method as a function of the ethanol content of the bulk solutions. Job's method followed by the application of the van't Hoff theory was used to determine the thermodynamic parameters of the molecular association. Results show quite different thermodynamics of formation of calixarene–amino acid complexes at low and higher ethanol content of the solutions. The considerable stability of the individual calixarene–aromatic amino acids complexes supports their existence also in the case when the amino acids are in a protein. To test this idea the conversion rate, enthalpy and entropy change associated to the structural transition of BSA (bovine serum albumin) were investigated by Differential Scanning Calorimetry (DSC) in the absence and in the presence of calixarene. Results show that presence of calixarene changes significantly both the thermodynamics and the kinetics of the transition of BSA and the information collected for the individual calixarene–amino acid complexes gives insights about the possible processes at molecular level.

Keywords Calixarene · Bovine serum albumin · Structural transition · Photoluminescence · Differential scanning calorimetry

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Introduction

Calixarenes are fascinating class of compounds which are able to form inclusion complexes not only with ions but also with small neutral molecules [1, 2]. Application of calixarenes in chemical sensors raises high interest since the steric hindering of the interacted species gives high selectivity character for the complex formation [3]. In our previous works the complexation behavior of calix[4]arene and 4-*tert*-butylcalix[6]arene (hosts) with pesticide related neutral π-electron deficient 1-trifluoromethyl-benzene derivatives (guests) in chloroform and dimethylformamide was reported [4, 5]. The effect of cavity shape [6] and solvent permittivity [7] on the complex formation were investigated in detail. However, further the properties mentioned above the role of the entropy was found to be one of the most important parameter regarding the complex stability [8]. This property is preferably based on the reordering of solvent molecules around the calixarene cavity under the effect of guest-induced polarization of calixarene aromatic rings [9, 10]. Furthermore, in the aqueous solution of ethanol both the thermodynamic [11] and kinetic properties [12] of the complex formation show significant change within a very narrow range of the solvent's composition. This property was associated with the unexpected change of the solvation shell, which idea was supported later by the solvent relaxation and rotational diffusion measurements [13].

In an early work Stone et al. showed [14] that calixarenes form complexes most of the amino acids, although they found the cavity of calix[4]arene is too narrow for complex formation with amino acids. However, functionalized calixarenes, such as calix[4]arene bis-hydroxymethylphosphous acid, form stable complexes with several amino acids [15]. The dependence of the interactions of calixarene with amino acids on the pH was also studied by

electrochemical impedance (EIS) and Fourier transform infrared spectroscopy (FTIR) [16].

Due to the dependence of the complex stability of calixarene–phenol complexes on the electron density of the aromatic moiety and also the unique property of the solvation shell exchange on the solvent composition in binary solvents it was obvious to examine the effect of presence of calixarene on the folding of proteins. Bovine serum albumin (BSA) which has all the three aromatic amino acids was selected to these studies.

Materials and methods

BSA was taken from Sigma–Aldrich (initial fraction by cold alcohol precipitation, Fraction V, $M_r \sim 66$ kD). Aromatic amino acids (Phenylalanine **2a**, Tyrosine **2b** and Tryptophan **2c**) taken from Reanal (Hungary). Thiacalix[4]arene-tetrasulfonate **1** was obtained by the *ipso* sulfonation of *p-tert*-butylthiacalix[4]arene as described in the literature [17]. The NMR spectra were recorded in D_2O (DSS standard) on a Bruker DRX-500 spectrometer. Structural characterization: ^{1}H NMR: δ 7.99 (σ, 8H. Ar*H*); ^{13}C NMR: δ 157.9, 132.8, 132.0, 118.30 (Ar*C*) (Fig. 1).

Both the fluorometric and the calorimetric experiments were carried out in the Sorensen buffer at pH $\sim$7.4/81 vol% 0.2 M disodium hydrogen phosphate (Merck) + 19 vol% 0.2 M sodium dihydrogen phosphate (Merck).

Fluorolog τ3 spectrofluorometric system (Jobin–Yvon/SPEX) was used to determine the thermodynamic parameters of interaction of calixarene with aromatic amino acids. For data collection a photon counting method with 0.2 s integration time was used. Excitation and emission bandwidths were set to 1 nm. 1 mm layer thickness of the fluorescent probes with front face detection was used to eliminate the inner filter effect.

To determine the thermodynamic parameters the Job's method was applied. Detailed information about this method is described elsewhere [10, 18]. Briefly, stock solutions of calixarenes and amino acids of 0.001 M were prepared in Sorensen buffer with pH = 7.4. To determine the stoichiometry of the complexes and the thermodynamic parameters of the complex formation, stock solutions of calixarene and stock solutions of the amino acids were mixed at eleven different molar ratios and the PL spectra were recorded. The ethanol content of the samples was varied in the range of 0 … 0.15 molar ratios in steps of 0.01. Data evaluation was performed using the HyperQuad software [19, 20].

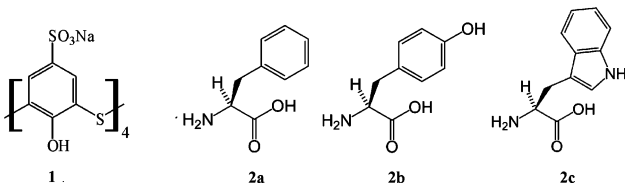

Fig. 1 Interaction of thiacalix[4]arene with three aromatic acid of BSA was investigated in this work

Calorimetric measurements were carried out with a nano-II-DSC 6100 (Setaram, France) instrument. The calorimeter is configured with a platinum capillary cell (volume = 0.299 mL). The samples were pressurized to $3 \pm 0.02 \times 10^5$ Pa during all scans. Using oil rotation pump, standard degassing procedure for 15 min at about 15 Pa was applied before loading the samples into the capillary. The heat flow was scanned between 0 and 78 °C. Scanning rate of 0.5, 0.75, 1.0, 1.5 and 2.0 K/min was applied to measure the transformation kinetics of the BSA. The experimental deviation of the calorimetric results was estimated to be $\pm$ 5 μJ.

The reaction rate was calculated by the Kissinger's method [21], where a plot of a function of heating rate and $\ln\left(\frac{H_r}{RT^2_{max}}\right)$ versus $1/T_{max}$ gives E_a/R from the slope and A/E_a from the intercept:

$$\ln\left(\frac{H_r}{RT^2_{max}}\right) = -\frac{E_a}{RT_{max}} + \ln\left(\frac{A}{E_a}\right)$$

Results

Table 1 summarizes the thermodynamic parameters about the interaction of calixarene (**1**) with three different aromatic amino acids (**2a**, **2b** and **2c**). Results show increased stability of the calixarene–amino acid complexes when the ethanol content of the samples is elevated to 0.12 molar ratio. Both in water and in the aqueous solutions of ethanol the order of the stability is the same: phenylalanine forms the strongest complexes with calixarene while the tryptophane–calixarene complexes are the most weakened. This is

Table 1 Thermodynamic parameters of interactions of calixarene 1 with amino acids **2a** … **2c**

Amino acid	ΔH (kJ/mol)	ΔS (J/K · mol)	ΔG (298.16 K) (kJ/mol)
Buffer			
2a	−46.4 (2)	−44.6 (3)	−33.10 (2)
2b	−48.1 (2)	−67.3 (3)	−28.03 (2)
2c	−49.3 (2)	−76.1 (3)	−26.61 (2)
Buffer + ethanol			
2a	−51.3 (2)	−40.7 (3)	−39.16 (2)
2b	−53.4 (2)	−61.8 (3)	−34.97 (2)
2c	−54.6 (2)	−88.6 (3)	−28.18 (2)

Solvents: *upper part*: phosphate buffer, *lower part*: as before but with ethanol of 0.12 molar ratio

in agreement with previous results where free enthalpy change is most pronounced when the electron density of the aromatic ring is the highest [8]. As an interesting property, the complex stability shows opposite tendency with the enthalpy change of the complex formation. This tendency was overcompensated with the entropy term as it also found earlier in cases of calixarene–phenol complexes [8, 10].

For study the possible effect of the calixarene on the conformation transition of BSA, we had to estimate an appropriate concentration of calixarene where most of the aromatic residue of the protein is covered by calixarene. To do that, we have determined the stability constants of the calixarene–amino acid complexes at the known transition temperature of BSA, which is around 58–59 °C. Figure 2 shows the stability constants of different calixarene–amino acid complexes as a function of the ethanol content of the samples. It can be clearly seen that according to the free-enthalpy change of the molecular association the phenylalanine–calixarene complexes associated with the highest stability constants. However, the stability of each complex shows unexpected increase at a well-defined composition of the solutions. Accordingly, the wide of ranges where the significant change overcomes are almost within 0.03 range of molar ratio. Furthermore, the phenylalanine show the unexpected increase of complex stability with calixarene at the lowest concentration ($x \sim 0.06$) of ethanol, then the stability of the tyrosine–calixarene complexes is increased ($x \sim 0.1$). Slight increase of tryptophane–calixarene complexes is also observed at $x \sim 0.13$. This interesting change in the stability of the complexes was discussed earlier in term of the far composition of the solvation shell from the composition of the bulk solution in water–ethanol [11] and other mixtures [13].

Now we have to consider the structure of BSA. It is known that further to other amino acids, BSA contains 30 phenylalanine, 21 tyrosine and 3 tryptophane amino acids. The common concentration of BSA used in the DSC measurements is 0.5 mM, i.e. 32.865 mg/mL prepared in the buffer solutions. Assuming non-restricted accessibility of all amino acid residues in the initial conformation of BSA and using the stability constants listed above, the fraction of the aromatic amino acids included by calixarenes can be as high as 98% when the concentration of the calixarene is chosen to 0.05 M. Note however, we assume interactions of calixarene only with the aromatic amino acids according to our measurements and no interaction is considered with other non-aromatic amino acids such as alanine or histidine although such interactions was reported in solid phase [22]. In contrast, in solution phase, especially at around pH $\sim$7 these interactions are showed to be reduced [14]. With this condition, the DSC curves were recorded and evaluated as described in the "Experimental" section.

The transition of BSA protein was characterized by three parameters: the enthalpy change, the entropy change (calculated as the ratio of the enthalpy and the transition temperature) and the activation energy of the transition. Two series of measurements were performed: the transition of the BSA were studied in the presence (Fig. 3) and in the absence of 0.05 M calixarene **1** (Fig. 4). In both cases the ethanol content of the solutions was changed from 0 up to 0.15 molar ratios and the thermodynamic parameters and the activation energies of the transition were plotted against the molar fraction of ethanol.

All thermodynamic data validate endothermic character of the protein transition in agreement with the literature. Furthermore, as it can be seen on Fig. 3, in presence of calixarene, the enthalpy and entropy change in pure water are 383 kJ/mol and 292 J/K · mol, respectively. The

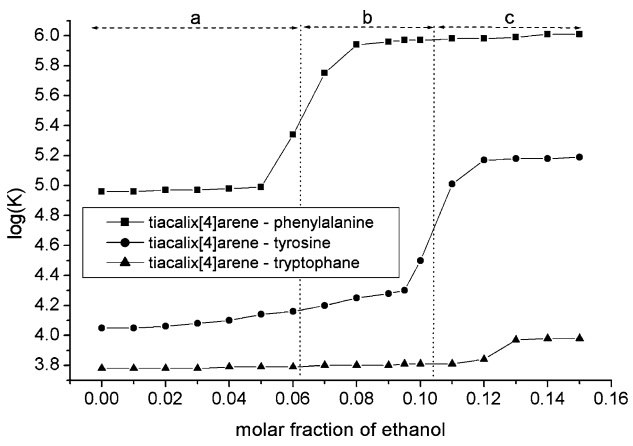

Fig. 2 Stability constants of calixarene–amino acid complexes as function of composition of the aqueous ethanol solutions. Data reflect stabilities for the transition temperature of BSA protein (59 °C)

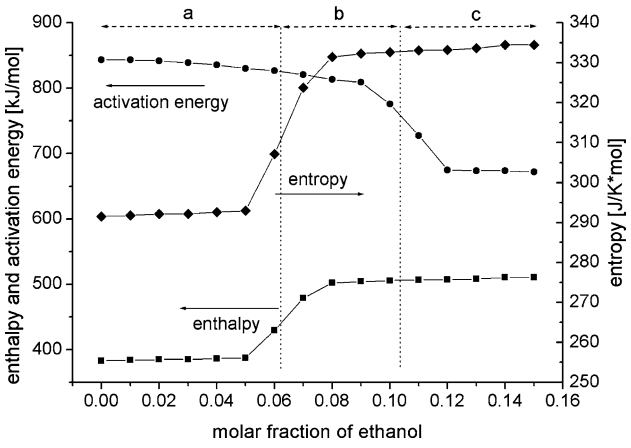

Fig. 3 Thermodynamic (ΔH and ΔS) and kinetic (E_a) parameters associated to the transition of BSA protein in the presence of 0.05 M calixarene **1**. The different molecular environment represented by aqueous solutions of ethanol where the ethanol content was varied within the 0 … 0.15 molar fraction

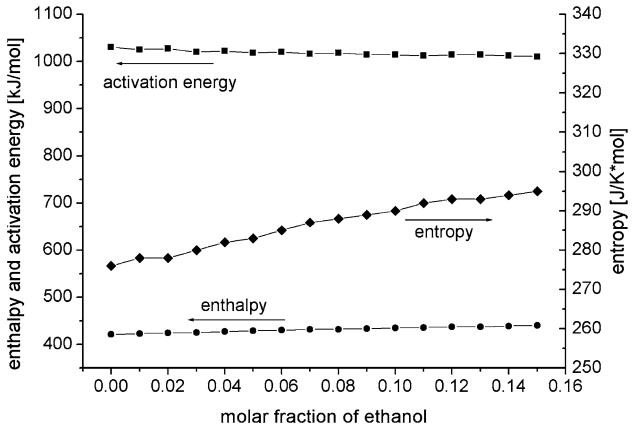

Fig. 4 Thermodynamic (ΔH and ΔS) and kinetic (E_a) parameters associated to the transition of BSA protein in the absence of calixarene. The different molecular environment represented by aqueous ethanol solutions where the ethanol content was varied within the 0 … 0.15 molar fraction

enthalpy value is a little lower compared to the appropriate value observed in the absence of the calixarene. This property probably due to the fact, that the calixarene includes some amino acids which, therefore, cannot participate anymore in the stabilization of the final state of the protein after its transition. This idea is supported with the entropy values, which highlights less improved disorder in the absence of calixarene. The activation energy is lower in the presence of calixarene molecules, maybe because these molecules slack off the protein structure prior the transition by weakening the interactions between the amino acids forming inclusion complexes with them. This process also could result the decreased enthalpy change observed in the presence of calixarene.

Increasing the ethanol content of the solutions, in the absence of calixarene slight increase of both the enthalpy and entropy term is obtained. This behavior can be resulted from the increased molecular interaction when the polarity of the solutions is lowered at their higher ethanol content. This change suggest pronounced role of the intramolecular interaction in the final state of the transition. As a parallel effect, the activation energy decreases with increasing the ethanol content.

All the tendencies discussed above remain valid in the presence of calixarene too. However, an unexpectedly large change is observed in the thermodynamic parameters (enthalpy and entropy change) when the molar ethanol fraction exceeds 0.06. No significant change is observed here on the activation energy. However, similar situation is found in the kinetic parameter (activation energy) when the ethanol content is around 0.1, where now the thermodynamic parameters do not show considerable change with the ethanol content. These two interesting molar ethanol fraction values, which are highlighted on the Figs. 2 and 3 by dotted lines, determine three characteristic regions of the ethanol content, where the calixarene affects the protein transition by three significantly different ways. To make conclusion on this observation we have to draw that the similar characteristic regions observed on the solution-composition dependent stability of calixarene–aromatic amino acid complexes (Fig. 2) are exactly the same compared to the solution-composition dependent transition properties of the BSA protein in the presence of calixarene (Fig. 3). Comparing the Figs. 2 and 3 to each other, the following molecular processes can be assumed adequately to the experiments: in the first range of ethanol content indicated as ‘a’ on the Figs. 2 and 3, the calixarene interacts with the amino acids and results some changes on the thermodynamic and kinetic parameters of the heat-induced conformational changes of BSA as it is described in detail at the beginning of this section. However, in range ‘b’ the interaction of calixarene with the phenylalanine is pronounced and also the enthalpy and entropy term associated to the transition of BSA are increased. Both parameters suggest the idea that phenylalanine participates rather in the stabilization of the final state of BSA and therefore the final state of the protein is destabilized by inclusion of phenylalanine units with calixarene. Reduced activation energy was measured in range ‘c’, where the calixarene–tyrosine interaction is pronounced. One description for this observation could be that most of the tyrosine amino acids are only accessible for the calixarene when the conformational change of BSA is in progress. During this process the interaction energy of the calixarene with tyrosine can be used to decrease the activation energy of the process. Since this pronounced calixarene–tyrosine complex formation doesn’t affect the thermodynamic parameters, the tyrosine probably do not participate in the stabilization of the final state of the BSA.

Conclusion

The effect of calixarenes on the structural transition of BSA was studied by DSC method. Result show significant changes both the thermodynamic and kinetic parameters of the transition under the presence of calixarene in the solutions. However, the variation of the molecular environment by changing the ethanol content of the aqueous buffer solutions performs further change on the thermodynamics. This property can be described by the significantly different complexation ability of calixarene towards the different aromatic amino acid in different molecular environment. The solvation shell exchange observed earlier was also detected in this particular case.

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