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Thermodynamic Aspects of Binding Proteins with Porphyrins. Spectral and Thermochemical Approaches

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The results of spectral and thermochemical studies of the interaction of bovine serum albumin with anionic and cationic porphyrins are presented in this paper. The limits of different methods for the determination of binding constants and thermodynamic parameters were shown. On the basis of spectral studies the binding constants of albumin with the porphyrins were evaluated. The fluorescence quenching constants of the protein at the porphyrins titration were estimated and it was found that in the albumin-porphyrin systems the static and dynamic quenching mechanisms are combined. The thermodynamic parameters were determined using of Van't Hoff equation from spectral data and the method of isothermal titration calorimetry. It was established that the stability constant of anionic complex is higher than that for complex of cationic porphyrin with albumin.

Keywords: Porphyrin, isothermal titration calorimetry, albumin, binding constant, fluorescence, viscometry.

Термодинамические аспекты связывания протеинов с порфиринами. Спектральный и термохимический подходы

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В работе различными методами исследовано взаимодействие бычьего сывороточного альбумина с анионными и катионными порфиринами. Показаны ограничения спектральных методов для определения констант связывания и термодинамических параметров.

Ключевые слова: Порфирин, калориметрия титрования, альбумин, константы связывания, флуоресценция, вискозиметрия.

Introduction

At present time, mechanisms and thermodynamic aspects of interaction of proteins and polynucleotides with the different specific ligands possessing fluorescent properties are intensively studied. These studies have theoretical and great practical importance, because the spectrum of practice for such substances is extensive. They can be used for diagnostic purposes – for assess the quantitative and qualitative changes in the structure of proteins under different physiological states of organisms,[1] for bioassay environmental components,[2] as vectors specific for specific DNA fragments,[3] as well as drugs for PDT.[4]

The structure of water soluble synthetic porphyrins is simpler than the structure of natural endogenous porphyrins (hematoporphyrin, protoporphyrin, uroporphyrin, *etc*.). That

is why the studies involving synthetic porphyrins can ensure a better understanding of the relationship structure–property. Different peripheral substituents in the porphyrin compounds priory affect their aggregation in aqueous media.[5,6] And spectral properties: electron absorption, fluorescence quantum yield of singlet oxygen, lifetime of the excited state, as well as localization and the binding strength of the porphyrin with biological substrates. The latter aspect is important in systems *in vivo*, because its will determine the distribution of porphyrin in bloodstream and safety of the drug and its dosage.

It is obvious that *in vitro* studies the problem of evaluation of efficient substrate binding with ligands is of paramount importance. Therefore the purpose of this study was a comparative analysis of various characteristics of binding efficiency of albumin with porphyrins. As objects of the study cationic and anionic porphyrins and bovine serum albumin were chosen. The studies were carried out in a borate buffer at p*H*=8.6.

Experimental

5,10,15,20-Tetrakis-(4-carboxyphenyl)porphyrin[7] and 5,10,15,20-tetrakis-(4-*N*-methylpyridyl)porphyrin tetraiodide[8] (H_2T((4-Me)NI)$_4$P) (Figure 1) were synthesized by the known methods in Ivanovo State University of Chemistry and Technology. The samples were dried under vacuum up to constant weight before use. The purity of the porphyrins was determined by ^{1}H NMR, MALDI-TOF and electronic absorption spectra.

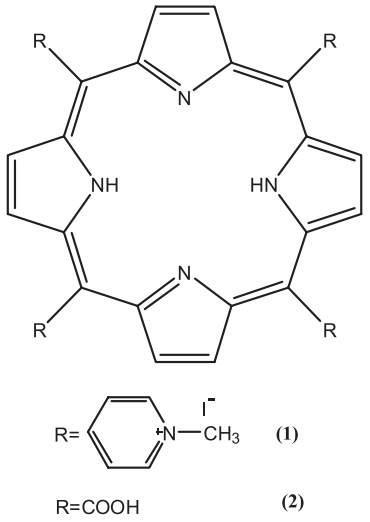

Figure 1. Chemical structure of porphyrins: 5,10,15,20-tetrakis-(4-*N*-methylpyridyl)porphyrin tetraiodide (porphyrin **1**), 5,10,15,20-tetrakis-(4-carboxyphenyl)porphyrin (porphyrin **2**).

Bovine serum albumin (BSA), fraction V, for biochemistry, p*H* 7.0 «Acros Organics» was used without additional purification. Borate buffer (p*H*=8.6) was prepared according to reference.[9]

The spectroscopic investigation was performed using a single-beam scanning spectrophotometer Unico 2800 (United Products and Instruments, Inc., USA) in the range of 200–800 nm. The fluorescence spectra were registered using a spectrophotometer "Avantes" AvaSpec-2048 in cell with a thermoregulation based on the Peltier element. Light source – xenon arc lamp with a diffraction monochromator LM-4 (Lumix).

The characteristics of complex formation were estimated according to Scatchard method[10] from fluorescence spectroscopic data. The mechanism of fluorescence quenching was determined by Stern-Volmer approach.[11] The thermodynamic characteristics were determined by the Van't Hoff equation.[12]

Molecular complex formation of the studied porphyrins with BSA was studied by isothermal titration calorimetry using a differential automatic titration calorimeter.[13] Calculation of thermodynamic characteristics of the complex formation has been reported by us in work.[14]

Results and Discussion

Differences in Literature Binding Constants

The interaction of H_2T((4-Me)NI)$_4$P with albumin has been widely studied, because of a significant increase in fluorescence intensity upon porphyrin binding to the protein globule. For example, the authors[15] reported that the binding constant of porphyrin **1** with BSA at p*H*=8.5 is $1.81\cdot10^6$ M^{-1}. According to[16] the binding constant of porphyrin **1** with BSA at p*H*=7.4 is $5.68\cdot10^5$ M^{-1}, while the binding constant reported in[12] is $1.73\cdot10^5$ M^{-1}. The maximum and minimum values of the binding constants of the porphyrin with albumin differ by one order of magnitude. What is the matter of such differences?

Let us consider the literature data. The authors[15] believe that the binding constant is the Stern-Volmer constant. Really, albumin fluoresces when irradiated with light due to the presence of fluorophores – tryptophan residues in 135 and 214 positions of polypeptide chain. Excited BSA* can be deactivated without emission and with emission of photon:

$$BSA + (295\ nm)h\nu \rightarrow BSA^* \rightarrow BSA + (340\ nm)h\nu$$

If BSA in ground state interacts with the porphyrin (H_2P) presented in solution and forms a complex:

$$BSA + H_2P \overset{K}{\rightleftarrows} BSA\cdot H_2P,$$

the fluorescence of BSA will decrease, and the concentration of free protein can be expressed as:

$$[BSA] = \frac{[BSA]_0}{(1 + K[H_2P])},$$

where $[BSA]_0$ is the initial concentration of BSA, K – equilibrium constant, $[H_2P]$ - concentration of porphyrin.

At irradiation all protein globules will absorb energy whereas only free globules will emit it, *i.e.*

$$\frac{F_0}{F} = 1 + K[H_2P], \quad (1)$$

where F and F_0 are the fluorescence intensities in the presence and absence of a quencher, respectively. In fact, the expression (1) is the Stern-Volmer equation. This equation can be applied only at static quenching

mechanism. In the case of a combination of dynamic and static quenching mechanisms, the equation becomes more complicated:[17]

$$\frac{F_0}{F} = (1 + K[H_2P]) \cdot (1 + k_q \tau_0 [H_2P]), \quad (2)$$

where τ_0 – lifetime of the excited state, k_q – quenching rate constant.

The authors of work[15] used equation (1) to determine binding constant without ascertainment of the quenching mechanism, therefore we carried out the appropriate spectral studies for determination of the binding constants. Figure 2 shows the fluorescence spectra of BSA in the presence of porphyrin **1**. At the titration of BSA by porphyrin **2** the spectra are similar.

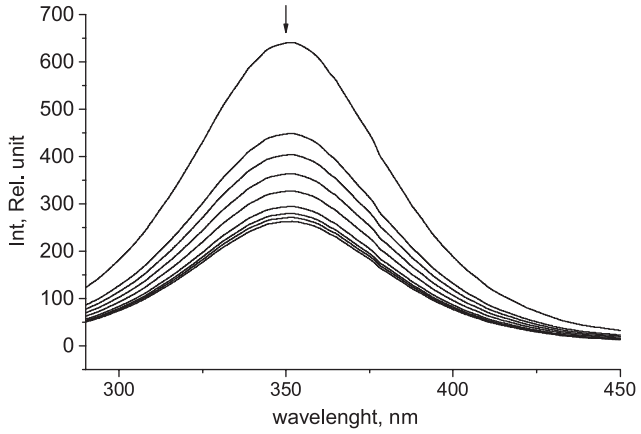

Figure 2. Fluorescence spectra of BSA in the presence of porphyrin **1**.

Obviously, the both porphyrins are quenchers of BSA fluorescence. In according to the isosbestic point in spectra of titration of studied porphyrin solutions (Figure 3.), in the ground state they form the complexes with BSA. Thus the static quenching occurs.

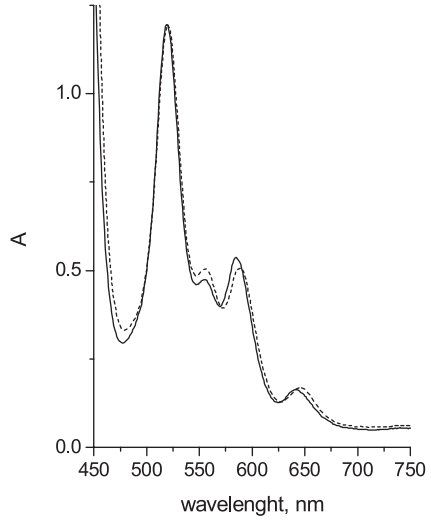

Figure 3. Electronic absorption spectra of porphyrin **1** (solid line) with BSA addition (dotted line).

Viscometry

The major requirement for dynamic quenching is the contact between fluorophore and quencher. The excited quencher, must diffuse to the fluorophore. The mean-square displacement $(\Delta x^2)^{1/2}$ of the quencher to the fluorophore during the lifetime in excited state (τ) can be calculated as:

$$\Delta x^2 = 2 \cdot D \cdot \tau,$$

where D is diffusion coefficient. The diffusion coefficient for the studied porphyrins was evaluated by the Stokes-Einstein equation (3), relating the diffusion coefficient with the particle radius (r) and viscosity of the medium (η)

$$D = \frac{k_B T}{6\pi r \eta}. \quad (3)$$

The viscosities of the BSA solutions, containing porphyrin **1** and porphyrin **2** in concentrations $(1–5)\cdot10^{-5}$ M in the borate buffer solution are 0.922 and 0.912 mPa·s, respectively. The porphyrin's lifetime in excited state in air saturated water solutions ($2.8\cdot10^{-4}$ mol·dm^{-3} O_2 at 25 °C at normal atmospheric pressure) is estimated 1.8 μs for porphyrin **1**[18] and 2.0 μs for porphyrin **2**.[19] The mean-square displacement at which the porphyrin tetra-ion can diffuse during the excited lifetime was 40 and 41 nm for **1** and **2**, respectively. The protein polypeptide chain forms globule in the crystalline state and in aqueous solution with p*H*=7.4. Its size is about 9 nm. However the globule can swell up to 80 nm depending on base electrolyte and p*H*.[20] In any case, the size of albumin is comparable with the displacement of the quencher. Thus, a probability of the dynamic quenching can not be excluded, especially in the case of porphyrin **1**, which can interact through electrostatic forces with the protein globules charged negatively at p*H*=8.6.

Fluorescence Quenching

According to the Stern-Volmer approach, quenching mechanism can be clarified at different temperatures. In the case of dynamic quenching, increasing temperature will increase the diffusion and the probability of molecules collision, which results in quenching increasing. At the static quenching mechanism, increasing temperature promotes the dissociation of the protein-quencher complex (with the exception of the complexes formed due to ionic electrostatic interactions), which leads to increase of fluorescence. Figure 4 shows the Stern-Volmer dependences (F_0/F via the quencher concentration) at heating the protein solution with cation and anion porphyrins.

As can be seen from Figure 4 the dependences are not strictly linear and their deviation from linearity increases with the increasing temperature, for both the cationic and anionic porphyrins. The obtained data suggest that in these systems both the static and the dynamic quenching mechanisms are realized. Therefore, the approach used by the authors[15] is not correct, and the constants need to be clarified.

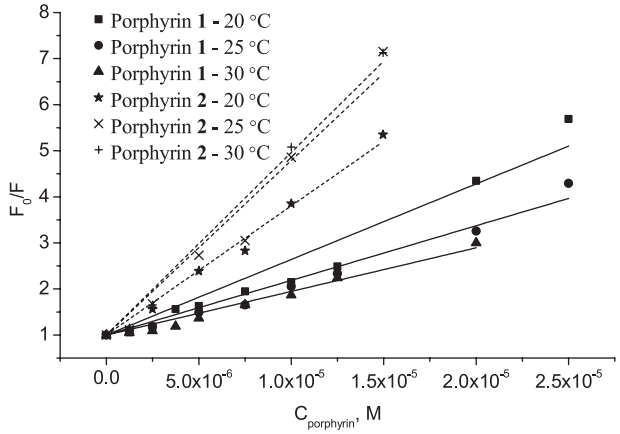

Figure 4. Stern-Volmer curves for quenching of BSA with porphyrins **1** and **2** at different temperatures.

The binding constants of porphyrins with BSA were determined by traditional or modified Scatchard, Hill equation. These approaches are based on the analysis of adsorption isotherms with subsequent linearization of equations. The following equations are usually used:

$$\lg\left[\frac{F_0 - F}{F}\right] = \lg K_a + n\lg\left[H_2P\right] \quad (4)$$

$$\lg\left[\frac{F_0 - F}{F - F_\varpi}\right] = n\lg\left[H_2P\right] - n\lg\left[\frac{1}{K_a}\right], \quad (5)$$

where $[H_2P]$ is the concentration of non complexed porphyrin.[21]

The constant K_a in the equations (4) and (5) is an empirical constant and its value is reciprocal of the concentration of free ligand occupied a half of the potential binding sites on the substrate. From the standpoint of physical chemistry this constant should be regarded as an empirical constant adsorption or the association constant. However, in a series of studies,[22-24] the constant calculated from the isotherms of binding is equated with the thermodynamic stability constant of albumin complexes with ligands. It is invalid viewpoint. Also it is not correct to consider the parameter *n* in equations (4) and (5) as molar composition of BSA-porphyrin complex.[25,26] The parameter *n* is maximum number of equivalent independent binding sites of BSA.

The source of possible errors in the determination of K_a is obvious: at plotting in Scatchard coordinates it is difficult to determine reliably the value of F_∞ or A_∞. It is particularly difficult in the case of the cationic porphyrins, which can bind electrostatically to the negatively charged surface of the albumin globule at p*H*>7 (isoelectric point of BSA is 4.6),[27] therefore saturation at the titration BSA-Porphyrin **1** is problematic. In addition, it is difficult to determine the concentration of free porphyrin, because the absorption spectra of free porphyrin and in the complex with the protein significantly overlap. Probably, last factor is the cause of the scatter of the data presented in[22,23] and Table 1.

Table 1. The association constants of BSA with porphyrins in borate buffer solution at different temperatures as calculated according to the equations (4)* and (5)**.

Compound	T, K	$K_a^* \cdot 10^{-5}$	n^*	R^2	$K_a^{**} \cdot 10^{-5}$	n^{**}	R^2
Porphyrin **1**	293	11.7	1.3	0.982	1.46[a]	2	0.989
	298	6.73	1.22	0.932			
	303	5.65	1.15	0.986			
Porphyrin **2**	293	7.50	1.08	0.998	2.81	1.26	0.978
	298	9.12	1.01	0.996			
	303	14.5	1.13	0.909			
	308	21.4	1.1	0.999			

[a] Published previously[28]

The obtained value of the association constant of Porphyrin **1** with BSA calculated according to equation (4) is consistent with the data reported in,[23] despite the fact that the studies were carried out at different p*H* values. Thermodynamic parameters of the investigated processes were determined from the temperature dependence of the association constants (Table 1) by the Van't Hoff equation (Table 2).

As can be seen from Table 2, the results obtained in this study disagree with the data reported in.[22] The probable reason is the composition of the base electrolyte and ionic strength from temperature.

Table 2. Thermodynamic characteristics of the association process of porphyrins with BSA in borate buffer at 298.15 K obtained using Van't Hoff equation.

Compound	*K*	Δ*G*, kJ·mol^{-1}	Δ*H*, kJ·mol^{-1}	Δ*S* J·mol^{-1}·K^{-1}
Porphyrin **1**	$1.05 \cdot 10^6$	–34.4	–54.19	–66.48
Porphyrin **1**[22]	$9.6 \cdot 10^4$	–27.4	–17.9	35.2
Porphyrin **2**	$9.80 \cdot 10^5$	–34.2	54.01	296

Furthermore, using of Van't Hoff equation for calculation of the thermodynamic parameters of protein containing solutions is limited. Firstly, the Van't Hoff approach is based on independence of the thermodynamic parameters on temperature. As mentioned above, secondly, the contributions from the static and dynamic fluorescence quenching of BSA at different temperatures will be different. Resulting changes of static and dynamic quenching is nonlinear dependence presented in Figure 4. Thirdly, the states of the reagents are significantly changed with the increasing temperature that is caused by the following reasons: BSA and the studied porphyrins are electrolytes and at the temperature increasing the dielectric constant of the aqueous solutions is reduced for 7 % per 10 °C.[29] The decrease of dielectric constant leads to reducing dissociation degree of the electrolytes and formation of contact and/or solvent-mediated ion pairs. This leads to an increase of the porphyrin's hydrophobicity and change of electron-donate ability of their peripheral substitutes. This is confirmed by decrease of intensity in the reflection spectra

Table 3. Thermodynamic parameters of complex formation cationic and anionic porphyrins with BSA in borate buffer (pH=8.6) at 298.15 K.

Compound	model	K	ΔG, kJ·mol^{-1}	ΔH, kJ·mol^{-1}	ΔS, J·mol^{-1}·K^{-1}	Sad
Porphyrin **1**	1:1	$7.05\cdot10^{3}$	–21.97±0.03	–37.62±0.48	–53±2	$2.99\cdot10^{-5}$
Porphyrin **2**	1:1	$5.38\cdot10^{4}$	–27.00±0.05	7.43±0.53	116±3	$5.19\cdot10^{-5}$

of BSA and electron absorption spectra of the porphyrin at heating. The decease of reflection spectra of BSA at heating indicating on decreasing size of the particulars in solution because the polyelectrolyte shrinks due to a greater neutralization of the amino acid residues of the polypeptide chain with increasing temperature (decreasing dielectric constant of the medium). The absorption spectrum of porphyrin **1** was practically unchanged during heating in borate buffer (pH=8.6). And optical density of porphyrin **2** in borate buffer increases significantly during heating. The charges of porphyrin's tetracation and tetraanion are more neutralized and this in turn, affects the associative equilibrium in solutions of porphyrins.

Isothermal Titration Calorimetry

The thermodynamic characteristics of complexation of porphyrins with BSA were determined by calorimetric titration. Solution of BSA in borate buffer was placed in the calorimetric cell and the solution of the porphyrin was placed in dozer. Heat effects, recorded at each step of the titration, adjusted to conform to the heat effects of single experiments, taking into account the effects of dilution of the porphyrin (porphyrin solution in a dispenser in the calorimetric cell – borate buffer) and protein (in the dozer – borate buffer, a calorimetric cell – BSA solution).

Albumin has several centers which can bind porphyrins, They are located in subdomains IIA and IB (I binding site by Sudlow[30] as well as heme site).[31] They have different structures and are located sufficiently far from each other. Therefore the obtained calorimetric titration curves were fitted with the following mathematical model of interaction:

$$BSA + H_2P \overset{K}{\rightleftarrows} BSA \cdot H_2P$$

$$BSA + 2H_2P \overset{K}{\rightleftarrows} BSA \cdot 2H_2P$$

$$BSA + 2H_2P \overset{K_1}{\rightleftarrows} BSA \cdot H_2P + H_2P \overset{K_2}{\rightleftarrows} BSA \cdot H_2P$$

In order to exclude possible local minima $\Sigma(Qn(\exp)-Qn(cal))^2$ function, profile of this function was plotted and analyzed for each system. The calorimetric titration curves are fitted very well by the model of monoligand complex formation. For the investigated systems the function $\Sigma(Qn(\exp)-Qn(cal))^2$ has a unique minimum, which was confirmed by the profile of the function (the profile was obtained by varying only one of the required parameters). Besides minimum of function testifies the greatest accordance between the calculated and experimental heat effects. The presence of single global minimum confirms the reliability of the obtained thermodynamic parameters. In Table 3 the thermodynamic parameters of the complexation of porphyrin **1** and porphyrin **2** with bovine serum albumin are presented.

The complexation of BSA with porphyrin **2** is endothermic, entropy-driven process. Hence, hydrophobic interactions are responsible for complex formation. In contrary, the complexation of BSA with the cationic porphyrin is exothermic and enthalpy controlled. That testifies about a significant contribution of electrostatic ionic interactions to the complex formation. The obtained data agree with the traditional conception of a higher affinity of the albumin binding site I to anionic heterocyclic ligands,[32] in comparison with cationic porphyrins.

Conclusions

Comparative analysis of the binding assessment of porphyrins with BSA was performed. It was found that in the case of a cationic porphyrin the results obtained by Scatchard, Stern-Volmer and classical spectrophotometric titration compared to isothermal titration calorimetry are significantly overstated. This is due to the fact that all spectral techniques imply the presence of saturation point, and in the case of cationic porphyrins it can not be achieved. This means that the cationic porphyrin reacts with BSA on the entire surface of the globules, which is negatively charged, in addition to binding sites.

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