Investigation of Interactions Between Cationic and Anionic Porphyrins and BSA in Aqueous Media

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Abstract In the present work interaction of the cationic and anionic porphyrins with bovine serum albumin in aqueous solutions with different pH has been studied. For strongly associated porphyrin study was conducted in a mixed solvent containing 0.19 M DMF, which shifted the associative equilibrium towards monomerization. It was found that the complexation of porphyrins with protein is possible due to π - π interaction between aromatic amino acid residues and π-system of porphyrin, hydrogen bonds between the peripheral substituents of porphyrins with amino acid residues of the protein, and in the case of sodium salt of 5,10,15,20-tetrakis (4-carboxymethylenoxyphenyl) porphyrin, ammonium salt of 5,10,15,20-tetrakis (4-sulfophenyl) porphyrin and 5,10,15,20-tetrakis (4-N-methylpyridyl) porphyrin tetra iodide interaction of amino acid residues of the protein to the reaction center of the porphyrin. It was determined that the complexation constants of studied porphyrins with protein are depends on the nature of the porphyrins peripheral substitution and does not depend on pH.

Keywords Porphyrins, Bovine Serum Albumin, Fluorescence, Stability Constants

1. Introduction

Albumins are the most abundant proteins found in blood. Albumin concentration in blood plasma and serum is higher the of other proteins. Albumin makes a main contribution to intravascular osmotic pressure. Moreover, the protein is a carrier of different biologically important substances [1]. Being detoxicant, albumin regulates hemostasis during transport of molecular compounds (ligands) which has low biologically active in bloodstream. It plays an important role in the transport and disposition of endogenous ligands (hormones, vitamins, fatty acids bilirubin, etc.) as well as endotoxins [2] and metabolites.

Binding function of serum protein is connected with its structural peculiarities including elements of recognition, structural adaptation and reversible fixation of biologically important substances [3]. Conformational alterations of albumin molecule due to irreversible binding of ligands, metabolites and toxins circulating in bloodstream at diseases result in reduction or abnormalities in physiological functions of the protein. In present time much attention is paid to development of express methods of detection of relationship between individual metabolites and their character and, hence, diseases. Investigations of binding ability of albumin are carried out using special molecules-fluorescent probes [4]. They occupy the sites of protein molecule meant for binding of endogenous metabolites. The increasing fluorescence of such sites allows to find the abnormalities of sorption ability of albumin.
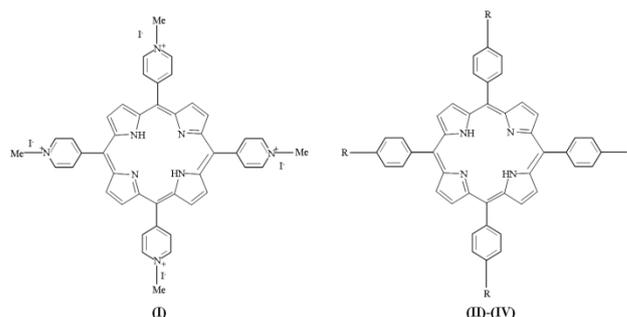

Figure 1. Chemical structure of porphyrins under study:

(I) 5,10,15,20-tetrakis (4-N-methylpyridyl) porphyrin tetra iodide (H_2T (4-NMePyI)$_4$P)
(II) R = N+ (CH$_3$)$_3$I-- 5,10,15,20-tetrakis (4-trimethylammoniyphenyl) porphyrin tetra iodide (H_2T ((4-Me$_3$)NI)$_4$P)
(III) R = OCH$_2$SOONa - Sodium salt of 5,10,15,20-tetrakis (4-carboxymethylenoxyphenyl) porphyrin (H_2T ((4-OCH$_2$COONa)$_4$P)
(IV) R = SO$_3$NH$_4$ - Ammonium salt of 5,10,15,20-tetrakis (4-sulfophenyl) porphyrin (H_2T ((4-SO$_3$)NH$_4$)$_4$P)

From chemical viewpoint, albumin is polyelectrolyte with isoelectric point pI= 4.6 that is negatively charged at physiological conditions. Albumin molecule contains several binding sites with different organization of inner cavity. For example, the inner walls of the pocket located in IIA subdomain are formed by hydrophobic side chains, whereas the entrance of the pocket is surrounded by positively charged residues of amino acids [5]. Therefore, anionic and cationic porphyrins may be considered as new

promising probes. The aim of the present spectroscopic investigation is to study peculiarities of interactions of series of porphyrins (Fig. 1) with bovine serum albumin (BSA) in aqueous media.

2. Materials and Methods

Studied porphyrins was purified by chromatography on Al_2O_3 (activity II). The sample was dried under vacuum up to constant weight before use. The purity of porphyrins was checked by electronic absorption spectra which demonstrated good agreement with literature data [6]. The purity of porphyrins was not less 99.98 %.

Bovine serum albumin (BSA), fraction V, for biochemistry, pH 7.0 «Acros Organics» was used without additional purification.

Dimethylformamide (DMF) were purified according to known procedures [7].

Acetate buffer (pH=4), phosphate buffer (pH=6.8) and borate buffer (pH=8.6) were prepared according to previous publication [8].

The spectroscopic investigation was performed using a single-beam scanning spectrophotometer Unico 2800. The fluorescence spectra were registered using a spectrophotometer Avantes AvaSpec-2048, a light source-Ivolga OMS-1 (Lumix) and a monochromator LM-4.

3. Results and Discussion

There are numerous studies in literature in which interactions between cationic and anionic porphyrins and proteins at different pH are considered from the viewpoint of electrostatic forces [9, 10]. Meanwhile main factors which determine character of formation of complexes such as ionic strength, effect of polyelectrolyte swelling, state of porphyrins in solution are neglected [10]. Taking into account results of our previous studies on influence of pH and electrolyte composition on association of macroheterocyclic compounds in solutions as well as effects of ionic strength of the solutions on conformational state of albumin [11], it is reasonable to study interactions between the porphyrins and BSA in wide pH range (from 4 to 8.6) in which the protein has native conformation [12].

Interaction of albumin with porphyrins has been studied in aqueous and aqueous-organic media containing 0.19 M DMF. Previously, with the use spectral and viscosity methods it has been found that DMF at concentration low than 0.2 M has no effect on the native conformation of the protein.

3.1. Acetate Buffer (pH=4)

As expected, solubility of anionic porphyrins in acetate buffer is very low due to protonation of peripheral substitutes (sulfo- and carboxyl- groups) of the porphyrins. In contrast,

cationic porphyrins have a significant solubility in the indicated medium. For $H_2T(4\text{-}NMePyI)_4P$ Bouguer-Lambert-Beer law is valid in the studied concentration range. This means that the porphyrin occurs in monomeric form. Deviation from Bouguer-Lambert-Beer law, that is broadening of Soret band as well as an inversion of intensities of II and III bands at increasing concentration of $H_2T((4\text{-}Me_3)NI)_4P$ in far wave region of electron absorption spectrum testifies about association of the porphyrin in acetate buffer. Porphyrins are hydrophobic molecules and their solubility in aqueous solutions is connected with ionization of peripheral substitutes. As a rule, dimer species are formed, and their stability in aqueous solutions results from π-π attraction and repulsion between likely charged peripheral substitutes of neighbouring porphyrin molecules. As can be seen from the molecular structures (Fig.1), $H_2T((4\text{-}Me_3)NI)_4P$ differs from $H_2T(4\text{-}NMePyI)_4P$ by the presence of more voluminous peripheral substitutes. However this structural peculiarity of $H_2T((4\text{-}Me_3)NI)_4P$ does not prevent coplanar interaction. Its inclination to association in aqueous media in comparison with $H_2T(4\text{-}NMePyI)_4P$ indicates on larger charge compensation of the peripheral substitutes that results in decreasing electrostatic repulsion of the substitutes and promotes self-association of the porphyrin. However at low concentrations (from $7\cdot10\text{-}6$ to $2\cdot10\text{-}5$ M) $H_2T((4\text{-}Me_3)NI)_4P$ is practically unassociated (Bouguer-Lambert-Beer law is valid for this concentration range). Therefore, stability constants of complexes of the porphyrins with BSA was determined from fluorescence spectra of the protein and the concentration of the porphyrins is within the indicated range. Table 1 shows the stability constants of the complexes.
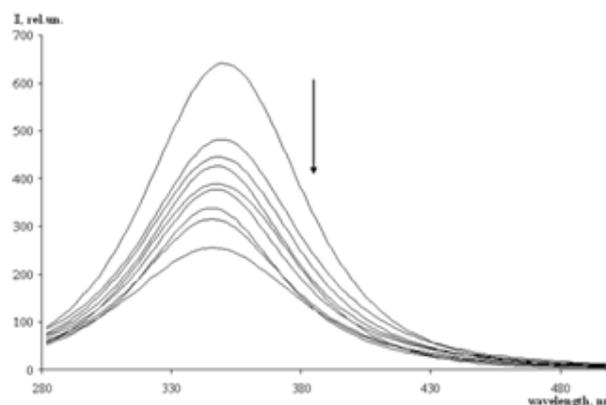

Figure 2. Changes of fluorescence spectra of BSA (0.08 % wt) at titration by $H_2T(4\text{-}NMePyI)_4P$ (from 0 to $3\cdot10^{-5}$ M) in acetate buffer (pH=4.0)

Figure 2 demonstrates typical spectrum of titration of BSA by $H_2T(4\text{-}NMePyI)_4P$. According to [13], fluorescence of BSA at λ=295 nm is connected with the presence of tryptophan residues in IB and IIA subdomains of the protein. Additions of the porphyrin results in decreasing fluorescence of BSA. This may be connected with either loss of native structure of the protein or energy transfer from the fluorophores (tryptophans) located in 214 and 135 positions

of the polypeptide chain to quencher (porphyrin). Absence of changes of fluorescence spectrum of $H_2T((4-Me_3)NI)_4P$ at addition of BSA is unexpected while the intensity of $H_2T(4-NMePyI)_4P$ fluorescence spectrum increases at addition of the protein. It is known from literature data [14] that growth of fluorescence of macroheterocyclic compounds at complex formation may be connected with removal of heavy counter-ions of peripheral substitutes. This fact can explains the increasing fluorescence of $H_2T(4-NMePyI)_4P$.   To elucidate the character of interaction between $H_2T((4-Me_3)NI)_4P$ and BSA, the spectrophotometric titration of the porphyrin in acetate buffer by the protein and tryptophan was carried out (Fig. 3).

The obtained spectra are similar. This shows that $H_2T((4-Me_3)NI)_4P$ interacts with BSA through π-π interactions between the aromatic system of macrocycle and tryptophan amino acid residue of the protein. The absence of changes of fluorescence spectrum of the porphyrin at addition of BSA is consequence of two opposite factors: 1) removal of iodide counter-ions promoting growth of fluorescence and 2) interaction between the porphyrin and tryptophan residue resulting in decrease of fluorescence. The thermodynamic constants of complex formation of BSA with $H_2T(4-NMePyI)_4P$ and $H_2T((4-Me_3)NI)_4P$ are found to be equal within the error (Table 1). Thus, only electrostatic interaction occurs between peripheral substitutes of the porphyrin and BSA which does not contribute significantly to the stability constants of BSA-porphyrin complexes.
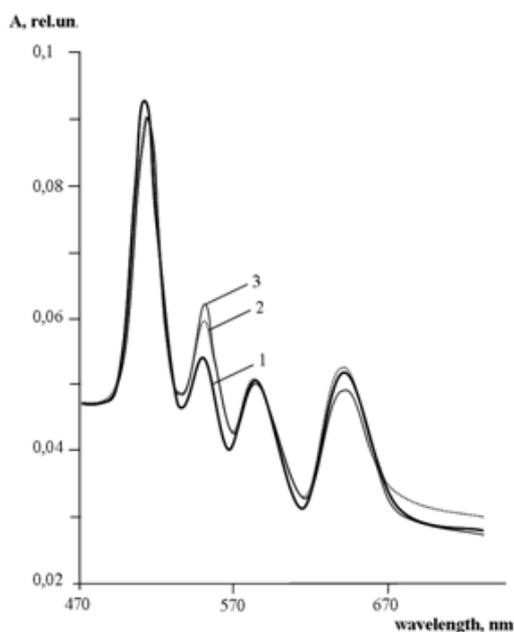

Figure 3.   Electron absorption spectra of $H_2T((4-Me_3)NI)_4P$ ($1 \cdot 10^{-5}$ M) (1); complex of $H_2T((4-Me_3)NI)_4P$ ($1 \cdot 10^{-5}$ M) and BSA (2); complex of $H_2T((4-Me_3)NI)_4P$ ($1 \cdot 10^{-5}$ M) and tryptophan (3) in acetate buffer (pH=4.0)

3.2. Phosphate Buffer (pH=6.8)

Transfer from acetate buffer to phosphate buffer results in dissolution of the studied anionic porphyrins $H_2T(4-OCH_2COONa)_4P$, $H_2T((4-SO_3)NH_4)_4P$ and $H_2T(4-NMePyI)_4P$. $H_2T((4-Me_3)NI)_4P$ is not soluble in these conditions. Similar to cationic porphyrins, the anionic porphyrins exhibit an intensive absorption of Soret band and in 500-700 range of electron absorption spectra. It should be noted that for the studied porphyrins except $H_2T(4-NMePyI)_4P$ a dependence of the absorption intensity on concentration is not linear. This testifies the association processes. Anionic porphyrins in phosphate buffer are inclined to association which occurs even at low porphyrin concentrations (up to 10^{-5} M). Therefore, to investigate interactions between the anionic porphyrins and BSA minimum concentration of the porphyrins were used. Calculation of the stability constants using Scatchard method was carried out from the fluorescence spectra of BSA. It should be taken into account that an additional association equilibrium (I) leads to an additional error in the stability constant values.

$$[P]_2 \leftrightarrow P+P \qquad\qquad (I)$$

$$P+BSA \leftrightarrow P \cdot BSA \qquad\qquad (II)$$
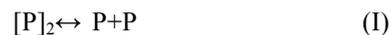
As it has been proposed in the previous work [15], in this case dimerization constants in two component system solvent- ligand should be estimated and correlation coefficient taking into account amount of monomer species of porphyrin should be introduced. Such approach is not thermodynamically correct because in three component system (solvent-protein-porphyrin) at least two interrelated equilibrium occur (I, II).

According to Le Chatelier's principle, an equilibrium (II) will shift to the right an equilibrium (I). Calculation of the thermodynamic characteristics of interrelated processes is not easy task. Therefore, to shift an equilibrium (I) to formation of monomer species, DMF (0.19 M) was added. As was earlier showed, such DMF concentration does not influence on the protein state in solution but shifts completely an equilibrium (I) to the right. The complex formation constants of BSA with the porphyrins are presented in Table 1. The obtained constant values are 3-5 times higher than the analogous values obtained with no account taken of an equilibrium (I). The stability of BSA-porphyrin complexes in phosphate buffer increases in the following order:

$H_2T((4-OCH_2COONa)_4P$ $\leq$ $H_2T((4-SO_3)NH_4)_4P$ $<$ $H_2T(4-NMePyI)_4P$ (III).
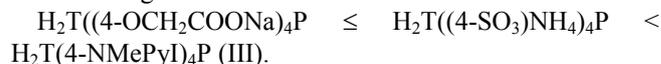
As has been mentioned above, dissolution of the anionic porphyrins in aqueous media occurs due to ionization of the peripheral substitutes. Taking into account that OCH_2COO^- group has a greater affinity to proton as compared with SO_3^- group, the position of $H_2T(4-OCH_2COONa)_4P$ in the order (III) is unexpected enough. The revealed regularity allows to suppose that the porphyrins located in IA and IIB subdomains of BSA are not protonated.
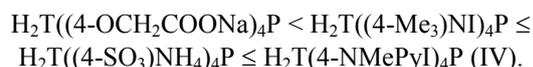
Table 1.   The stability constants (K± 3% (l/mol)) and the number of binding sites (n) of porphyrins with BSA in a variety of environments, calculated by the method of Scatchard

Porphyrins	Acetate buffer (pH=4)		Phosphate buffer (pH=6.8)		Neutral medium (NaCl solution, 0.05M)		Borate buffer (pH=8.6)	
		*DMF		*DMF		*DMF		*DMF
$H_2T(4\text{-}NMePyI)_4P$	n=2 K=1.27·10⁵		n=1.4 K=1.46·10⁵		n=2.2 K=2.94·10⁵		n=2.1 K=1.46·10⁵	
$H_2T((4\text{-}Me_3)NI)_4P$	n=1 K=1.14·10⁵	n=1.4 K=1.43·10⁵	not soluble		not soluble	n=2 K=1.27·10⁵	not soluble	
$H_2T((4\text{-}SO_3)NH_4)_4P$	not soluble		n=2 K=1.6·10³	n=1 K=8.62·10⁴	not soluble	n=2 K=1.89·10⁵	n=1.6 K=2.13·10⁴	n=2.3 K=1.24·10⁵
$H_2T((4\text{-}OCH_2COONa)_4P$		n=0.7 K=2.43·10⁴	n=1 K=2.03·10⁴	n=2 K=7.18·10⁴	not soluble	n=2.3 K=2.79·10⁴	n=1 K=4.12·10⁴	n=1.4 K=3.3·10⁴
*constants are defined in the environment of the mixed solvent containing 0.19 M DMF								

3.3. Neutral Medium (NaCl Solution, 0.05M)

At the investigation of complex formation of BSA and the porphyrins in water NaCl (0.05M) was added to overcome the effect of polyelectrolyte swelling, that is constant alterations of the protein conformation in solution at low ionic strength [16].

All the porphyrins studied except $H_2T(4\text{-}NMePyI)_4P$ are not soluble in aqueous NaCl solution without DMF additions. Therefore, the investigations were carried out in the mixed solvent: H_2O – NaCl (0.05 M) – DMF (0.19M). The additions of all porphyrins in BSA solution results in quenching of the protein fluorescence (λ_{ex}=295 nm). The calculated stability constants of BSA-porphyrin complexes are presented in Table 2. The stability of the complexes in aqueous NaCl solutions increases in order:

$$H_2T((4\text{-}OCH_2COONa)_4P < H_2T((4\text{-}Me_3)NI)_4P \leq$$
$$H_2T((4\text{-}SO_3)NH_4)_4P \leq H_2T(4\text{-}NMePyI)_4P \text{ (IV)}.$$

3.4. Borate Buffer (pH=8.6)

pH Value of borate buffer is maximum at which native conformation of BSA is kept. The increase of pH promotes a shift of the equilibrium to formation of monomer species of the anionic porphyrins. As in other buffers, the stability constants of BSA-porphyrin complexes were determined by Scatchard method from the spectra of quenching of the protein at additions of the porphyrins. A lesser difference between the stability constants of the complexes determined in presence and absence of DMF can be explained by a greater amount of monomer species of the anionic porphyrins in borate buffer in comparison with phosphate buffer. The quenching of fluorescence of BSA at 340 nm (Fig. 4) indicates on binding of the porphyrins with IA and IIB sites of the protein.

The stability of the formed complexes in borate buffer increases in the following order:

$$H_2T((4\text{-}OCH_2COONa)_4P < H_2T((4\text{-}SO_3)NH_4)_4P \leq$$
$$H_2T(4\text{-}NMePyI)_4P \text{ (V)}$$
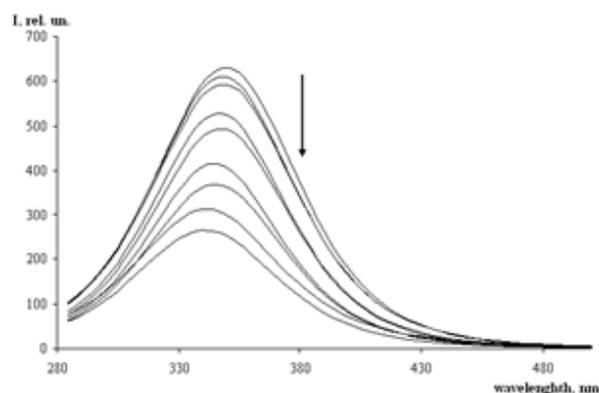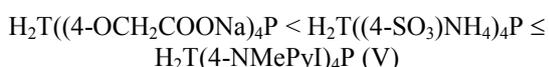
Figure 4.   Changes of fluorescence spectra of BSA (0.08 % wt) at titration by $H_2T(4\text{-}NMePyI)_4P$ (from 0 to 2·10⁻⁵ M) in borate buffer (pH=8.6)

It should be noted that the order of increasing stability of complexes of BSA and the porphyrins is the same in the studied media. Besides, the stability constants of complexes of each porphyrin with BSA in different media are equal within the error. This fact allows to conclude that the ionic strength and pH value of the media do not influence significantly on the complex formation process. The obtained data confirm indirectly intercalation of the porphyrins into IA and IIB hydrophobic sites of the protein. The stability constants of BSA - $H_2T(4\text{-}OCH_2COONa)_4P$ complexes is a order of magnitude lower than analogous values for other complexes. It is likely there are certain steric requirements to ligands for their inclusion into tryptophan-containing sites of IA and IIB subdomains. The data of fluorescence spectra of the porphyrins at different wave lengths of excitation are presented in Table 2. As can be seen from Table 2, ratio of intensities of the bands in fluorescence spectra of the porphyrins depends on wave lengths of excitation for all the studied systems except $H_2T(4\text{-}NMePyI)_4P$ in phosphate buffer. According to previous works [17] this can be due to an additional contribution from N-H-N tautomeric equilibrium in the fluorescence spectra of the porphyrins at irradiation by light of far-wave region. At addition of BSA to solutions of the all

studied porphyrins except $H_2T((4-Me_3)NI)_4P$ an essential change of the ration of intensities of fluorescence bands after excitation at 500 nm is observed. Thus, it can be concluded that besides the aromatic system of the macrocycles, their reaction center are involved in the complex formation of BSA with the porphyrins. This leads to "exception" of protons of the reaction center from the tautomeric equilibrium.

The intensive fluorescence of the porphyrins in complexes with the protein at irradiation by light of far-wave region has

an important practical application because the porphyrins can be used as fluorescence probes. On the basis of the obtained data on stability of complexes of BSA with the studied porphyrins (Table 1) and changes of spectral characteristics of the porphyrins in the complexes with BSA (Table 2) it can be concluded that $H_2T(4-NMePyI)_4P$ and $H_2T((4-SO_3)NH_4)_4P$ are the most promising for this application. These porphyrins form the most stable complexes with BSA and change essentially their spectral characteristics in comparison with other studied porphyrins.

Table 2. The position and intensity of the maximum of the fluorescence spectra of porphyrins and their complexes with BSA in different media at an excitation wavelength of 450 and 520 nm

Porphyrins and complexes	Acetate buffer (pH=4)		Phosphate buffer (pH=6.8)		Neutral medium (NaCl solution, 0.05M)		Borate buffer (pH=8.6)	
excitation wavelength	450 nm	520nm	450nm	520nm	450nm	520nm	450nm	520nm
$H_2T(4-NMePyI)_4P$	664(1521)/ 706(1326)	666(1536)/ 705(1507)	670(823)/ 703(860)	670(879)/ 705(917)	670(685)/ 704(827)	671(830)/ 705(846)	667(1060)/ 701(898)	670(780)/ 704(801)
$H_2T(4-NMePyI)_4P+BSA$		662(1073)/ 707(930)		670(744)/ 705(746)		667(870)/ 706(831)		667(845)/ 705(760)
$H_2T((4-Me_3)NI)_4P$	674(2512) shoulder	652(2150)/ 701(1332)			not soluble			
$H_2T((4-Me_3)NI)_4P+BSA$		652(2151)/ 700(1332)						
$H_2T((4-SO_3)NH_4)_4P$	not soluble		662(496)/ 701(310)	647(3680)/ 700(1920)	678(2438) shoulder	646(3630)/ 700(2440)	656(525)/ 703(306)	647(4737)/ 701(2473)
$H_2T((4-SO_3)NH_4)_4P+BSA$				654(3930)/ 718(1531)		651(4672)/ 710(1985)		655(1714)/ 717(929)
$H_2T((4-OCH_2COONa)_4P$	not fluoresce						650(593)/ 704(220)	651(1759)/ 704(773)
$H_2T((4-OCH_2COONa)_4P+BSA$								653(2589)/ 761(811)
The data is presented in a format λ, nm (intensity of fluorescence)								

4. Conclusion

Thus, the results obtained in the present work demonstrate that

i.   when self-association of macroheterocyclic compounds in solutions occurs, their interactions with protein should be carried out in mixed water-organic solvent, containing an amount of DMFA which is necessary to shift the association equilibrium towards formation of monomer species but does not cause negative effect on the protein

ii. the stability constants of complexes of BSA with the porphyrins studied are not practically affected by a nature of peripheral substitutes of the porphyrins and pH. The stability of the complexes is determined by hydrophobic, π-π interactions between aromatic amino acid residues and π-system of the porphyrin macrocycle, weak hydrogen bonding between the peripheral substitutes of the porphyrins and amino acid residues of BSA. In the case of $H_2T(4-NMePyI)_4P$, $H_2T((4-SO_3)NH_4)_4P$ and $H_2T(4-OCH_2COONa)_4P$ the interactions between amino acid residues of the protein and the reaction center of the porphyrins are involved in the complex formation.

iii. in contrast to an opinion about absence of stereoselectivity of binding of ligand with albumin, by way of example of $H_2T(4-OCH_2COONa)_4P$ it was shown that structural adaptation of BSA has definite parameters in the case of intercalation of the macroheterocyclic compounds into subdomains IA and IIB of the protein

iv. $H_2T(4-NMePyI)_4P$, $H_2T((4-SO_3)NH_4)_4P$ are promising compounds for their application as fluorescent markers for analysis of sorption capability of binding sites in IA and IIB subdomains of BSA because these porphyrins fluoresce intensively and form the most stable complexes with the protein.

Acknowledgements

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