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Fluorescence Study of Sinapic Acid Interaction with Bovine Serum Albumin and Egg Albumin

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The mechanism of interaction of protein with compounds used for preparation of matrices for matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS) methods is unknown. This paper reports the investigation of this mechanism for sinapic acid and bovine serum albumin and egg albumin. To examine these interactions in water a fluorescence method was applied. Sinapic acid can exist in three different forms, depending on pH: undissociated and with one or two deprotonated groups. pK_as of these states are: 4.47 for the COOH group and 9.21 for the OH group [1]. Therefore the interactions were examined at pH: 2.0, 6.4, and 10.5. The results show that sinapic acid at pH 10.5, being a bivalent anion, does not form any complex with these two proteins. At pH 2.0, sinapic acid, being undissociated, interacts weakly with egg albumin. Sinapic acid does not interact with bovine serum albumin at this pH. At pH 6.4, sinapic acid interacts only with bovine serum albumin. Parameters of the sinapic acid and bovine serum albumin complex were calculated based on the theory of multiple equlibria: the total number of binding sites, N = 15; the binding constant, K = 600 M^{-1}; and the Hill's coefficient, j = 0.97. These parameters indicate (but not definitively because a large saturation was not obtained) that this is a simple binding of sinapic acid to bovine serum albumin with the binding sites of the same type.

KEY WORDS: Sinapic acid; flourescence; ligand-protein interaction; pH.

INTRODUCTION

Since 1988, work has been developed on matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS), a new method for mass spectrometry of proteins. The search continues for matrix compounds. Cinnamic acids and benzoic acids seem to be good candidates for matrix-forming compounds. Sinapic acid (SA) has been used as a one of the main compounds for matrix formation [2–4]. To prepare a matrix with protein, the protein solute is added to the matrix compound solution, and then some aliquot of solution is dried and may crystallize. All that happens during the process of drying or crystallization has a decisive influence on the matrix with protein. The interaction of proteins with compounds used for matrix formation in solution and during the process of crystallization affects the protein distribution in the matrix. It seems that the complex should not dissociate during the process of drying and crystallization because lyophilized proteins exhibit "pH memory," that is, their behavior in the solid form corresponds to the pH of the aqueous solution from which they were freeze dried [5]. The pK_a values in the aqueous and lyophilized states for each compound containing groups that can dissociate in protein were found to be similar [5]. However, it is necessary to be cautions, because local pH may be dramatically changed depending on the buffer used [6]. In the dried and frozen states, proteins undergo deterioration that may be both chemical and conformational [7]. Their secondary and quaternary

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ABBREVIATIONS: SA, Sinapic acid; BSA, bovine serum albumin; EggA, egg albumin.

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structure can be changed, which may affect interaction with SA in the matrix.

It is important to investigate these interactions in solution. All cinnamic and benzoic acids can dissociate in water solutions. The equilibrium between the dissociated and undissociated forms will shift according to the concentration of compound and solvent pH. The number of water molecules left in the matrix after crystallization is unknown, so changes of pH may cause the change of absorbance wavelengths. This in turn will affect the value of absorbed energy and effectiveness of the MALDI method. Beavis and Bridson [2] found that horse skeletal muscle myoglobin and bovine serum albumin interact hydrophobically with sinapic acid in the matrix [2]. The investigations by Strupat *et al*. [8] with chicken egg white lysozyme and horse heart cytochrome *c* is in contradiction to this finding. They believed that hydrophobicity of one planar surface is not a prerequisite for protein binding and a variety of different noncovalent interactions that lead to incorporation of protein into matrix crystals should be considered. A quantified incorporation of the protein into a matrix is unknown, so more detailed study is warranted.

The aim of this study was to examine the type of interactions of different sinapic acid forms with proteins, bovine serum albumin (BSA) and egg albumin (EggA), that may lead to complex formation. The analyses were carried out with the use of absorption and steady-state fluorescence methods. The general theory of multiple equilibria in proteins [9–13] was used for calculating the parameters of the complex.

EXPERIMENTAL

Materials

Sinapic acid (SA) was obtained from FLUKA and quinine sulphate from Sigma (St. Louis, MO, USA). Bovine serum albumin was ordered from BDH England and egg albumin from CALBIOCHEM. Analytical grade reagents were obtained from POCH Gliwice (Poland). The Britton-Robinson universal buffer solution (pH 2–12) and carbonate-sodium bicarbonate buffer solution (pH 10.5, 0.1 *M*) were used as solvents.

The SA stock solution for measurements in function of pH was prepared in redistilled water and was diluted with buffer to the volume ratio of 1:4. The final concentration of SA was 10^{-4} *M*.

The SA solution ($2 \cdot 10^{-3}$ *M*) for titration and solutions of BSA and EggA ($8 \cdot 10^{-5}$ *M*) were prepared in the Britton-Robinson buffer. Molar ratios of BSA and EggA to SA ranged from 1:25 to 5:1. Buffer solutions of BSA + SA for study in the function of BSA concentration were prepared in a similar way, with the ratio of concentration of BSA/SA from 1:30 to 1:3. The BSA concentration was determined spectrophotometrically, taking $E^{1\%}_{1cm} = 6.67$ at $\lambda = 280$ nm and $M_r = 67{,}000$ [14]. The value of $E^{1\ g/100\ ml}_{1\ cm} = 7.12$ at $\lambda = 280$ nm and $M_r = 43{,}000$ were used for EggA concentration determination [15].

Methods

Theory Used

Ligand binding with proteins can be described by [9–13]:

$$\tilde{n} = \frac{N \cdot K \cdot [L]}{1 + K \cdot [L]} \tag{1}$$

where ñ is the average number of bound protein-ligand molecules at independent and equivalent sites, N is the maximum number of binding sites on protein, K is the binding constant, and [L] is the equilibrium concentration of free ligand in the solution. If there are more than one type of protein binding sites, the following equation should be used:

$$\tilde{n} = \frac{N_1 \cdot K_1 \cdot [L]}{1 + K_1 \cdot [L]} + \frac{N_2 \cdot K_2 \cdot [L]}{1 + K_2 \cdot [L]} + \ldots + \frac{N_N \cdot K_N \cdot [L]}{1 + K_N \cdot [L]} \tag{2}$$

with the assumption that the conformation changes of protein do not occur as the result of ligand binding. The results can be presented on the direct (binding isotherm), semilogarithmic, Scatchard, or reciprocal plot [10,16,17]. The nature of binding can be judged from the Hill's coefficient (j), obtained from [18]:

$$\mathrm{Log}\left[\frac{\tilde{n}}{N - \tilde{n}}\right] = \mathrm{Log}[K \cdot [L]]^{+j} \tag{3}$$

The value of $j = 1$ indicates the case of a simple binding, $j < 1$, represents a cooperative-positive binding, and $j > 1$ is a multiple binding. In the case of a cooperative-positive binding, Eqs. (1) and (2) cease to be valid.

To determine the complex parameters, [L] and ñ have to be calculated. In this paper, the method based on fluorescence quantum yield is applied:

$$Q_r = Q_f \cdot \frac{[L]}{[L_0]} + Q_b \cdot \frac{[PL]}{[L_0]} \tag{4}$$

where Q_r is the fluorescence quantum yield of the ligand-protein solutions; Q_f, the fluorescence quantum yield of free ligand; Q_b, the fluorescence quantum yield of totally bound ligand; Q_p, the fluorescence quantum yield of free protein; Q_{pb}, the fluorescence quantum yield of bound protein; [L], the concentration of free ligand; [PL], the concentration of bound ligand; $[L_0]$, the total concentration of ligand; and $[P_0]$, the total concentration of protein.

The fluorescence quantum yield of a sample was determined according to:

$$Q_s = Q_w \cdot \left[\frac{f_p}{f_w}\right] \cdot \frac{1 - 10^{-D_w}}{1 - 10^{-D_p}} \quad (5)$$

where Q_s is the fluorescence quantum yield of a sample; Q_w, the fluorescence quantum yield of a standard (known); f_p, the amount of quanta emitted by a sample (measured); f_w, the amount of quanta emitted by a standard (measured); D_w, the absorbance of a standard for a wavelength used for excitation (measured); and D_p, the absorbance of a sample for the same wavelength as that used for a standard (measured).

If the absorbance of a sample equals the absorbance of a standard for a given wavelength, then Eq. (5) is reduced to:

$$Q_p = Q_w \cdot \left[\frac{f_p}{f_w}\right]_{D_p = D_w} \quad (6)$$

To calculate $[PL] = f\,[L_0]$ and $[L] = [L_0] - [PL] = [L_0] - f \cdot [L_0] = [L_0]\,(1\text{-}f)$ it is necessary to know *f*. The parameter *f* can be calculated from:

$$f = \frac{Q_r - Q_f}{Q_b - Q_f} \quad (7)$$

Obtained values can be used for calculation of ñ:

$$\tilde{n} = \frac{[PL]}{[P_0]} = f \cdot \frac{[L_0]}{[P_0]}$$

and plot formation of binding isotherm.

To obtain the value of *f* from the experimental data, one should find Q_b, which can be determined by titration of ligand with protein. The data from titration can be presented in the plot of $1/Q_r$ versus $1/[P_0]$ and extrapolated to infinite protein concentration. Pesce *et al.* [19] suggest that this relation should be linear. Hence the value of Q_b can be determined by extrapolation of the data presented in a coordinate system $1/Q$, $1/[P_0]$, where $[P_0]$ stands for the total concentration of protein introduced to the solution. The point of intersection with $1/Q$ axis shows the searched value of $1/Q_b$.

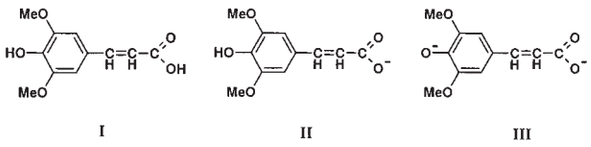
The constant (or constants) of a binding, as well as N—the maximum number of binding sites, can be determined by titration of protein with ligand.

Procedure

The fluorescence spectra were measured with the use of frontal excitation in a 0.2-cm cell in a homemade setup described in [1] and were corrected for reabsorption. Three wavelengths for excitation were used: 300, 333, and 365 nm. It was assumed that $Q_w = 0.50$ of quinine sulphate for 300 and 333 nm, and 0.55 for 365 nm [20]. The absorption spectra were registered with a spectrophotometer (Cary 3E [Varian]). The experiments were performed at temperature of 24 ± 1°C. Data were calculated using the computer program Prism 2.01 (GraphPad Software Incorporated).

RESULTS

Sinapic acid can exist in three forms: an undissociated form (form I), as a monovalent anion with a dissociated COOH group (form II), and as a bivalent anion with a dissociated COOH and OH group (form III).



The absorption spectra of SA forms are presented in Fig. 1 [1]. As pH of the solution increases, the second

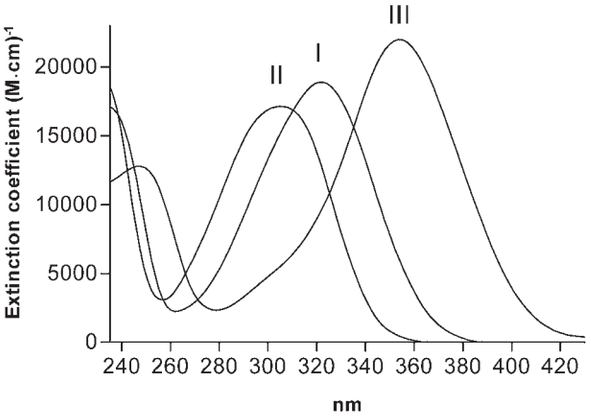


Fig. 1. Absorption spectra of ionic forms of sinapic acid. I, Undissociated; II, with dissociated COOH group; III, with dissociated COOH and OH groups.

maximum appears at fluorescence spectra [1], centered at 479 nm for $\lambda_{exc} = 333$ nm. Instead, only one fluorescence band is observed for the second excitation wavelength, $\lambda_{exc} = 365$.

The pK_a values of SA for each form in the excited states are for the COOH group 4.50 ± 0.04 and for the OH group 9.13 ± 0.07. These values are the same in the ground state [1]. The values of pK_a in excited state were determined using Förster-Weller's cycle [21]. The changes of fluorescence quantum yield obtained for SA solutions are shown in Fig. 2.

The experiments with BSA and EggA were carried out for three pH levels: 2.0, 6.4, and 10.5. At pH 2.0, SA exists as an undissociated molecule (form I); at pH 6.4 SA the carboxyl group is almost dissociated (form II). At pH 10.5, SA exists mainly as a bivalent anion with an almost completely dissociated hydroxyl group (form III). The amount of two forms in the ground state can be calculated for a proper pH, having pK_a for each form, from the formulae:

$$[\text{form I,II}] = [c] \cdot \frac{10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}}$$

$$[\text{form III}] = [c] \cdot \frac{10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}}$$

where [c] is the concentration of SA. The results of calculations are shown in the Table 1.

The Fluorescence Spectra of SA in the Presence of Proteins at pH 6.4

The samples were excited with $\lambda_{exc} = 333$ and 365 nm. Upon adding BSA to SA solutions at pH 6.4 and with increasing concentration of SA, the intensity of fluorescence increased and the band maximum shifted toward longer wavelength, to 463 nm (Fig. 3, $\lambda_{exc} = 333$ nm). Figure 4 shows fluorescence of the same solutions, excited at wave-

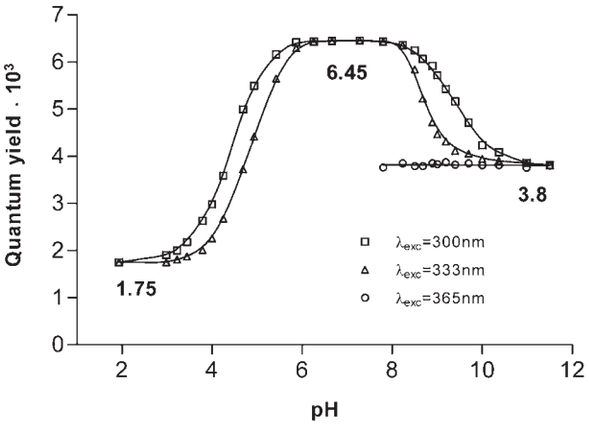


Fig. 2. Fluorescence quantum yield of sinapic acid for different pH and for different λ_{exc}.

Table I. The Amount of SA Forms in the Ground State Calculated for Different pH Levels

Forms	pH 2.0	pH 6.4	pH 10.5
I	99.7%	1.2%	–
II	–	98.6%	4.9%
III	–	0.2%	95.1%

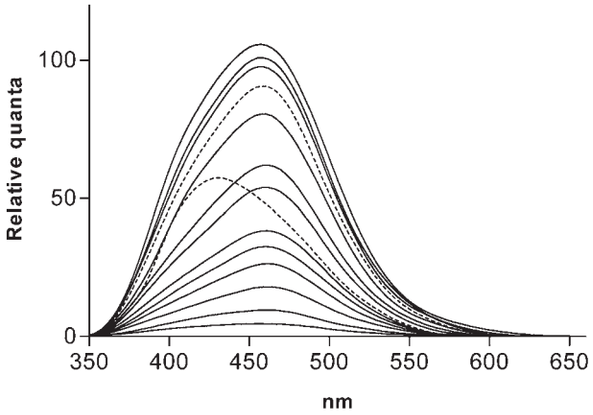


Fig. 3. Fluorescence spectra of sinapic acid–bovine serum albumin solutions (excitation at 333 nm, pH 6.4) with increasing concentration of bovine serum albumin. Broken lines indicate the same concentrations of sinapic acid with (upper curve) and without (lower curve) bovine serum albumin.

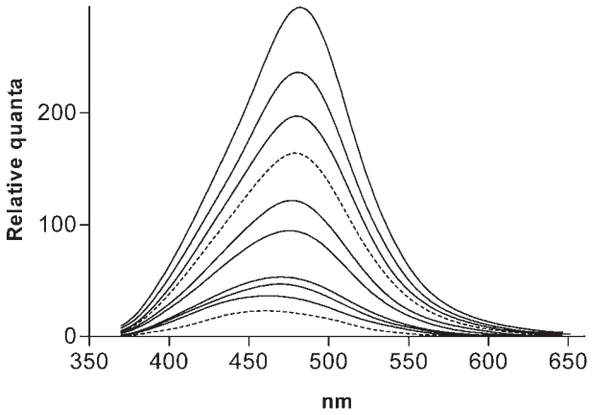


Fig. 4. Fluorescence spectra of sinapic acid–bovine serum albumin solutions (excitation at 365 nm, pH 6.4) with increasing concentration of bovine serum albumin. Broken lines indicate the same concentrations of sinapic acid with (upper curve) and without (lower curve) bovine serum albumin.

length λ_{exc} = 365 nm. Spectra of SA + BSA are more shifted toward longer wavelengths (~480 nm) than those excited with λ_{exc} = 333 nm. The increase in the fluorescence intensity and quantum yield were also larger then for λ_{exc} = 333 nm. Thus, although the amount of form III in solutions at pH 6.4 is very low (Table I), the contribution of its emission cannot be neglected for longer-wavelength excitation.

Summarizing, addition of BSA to SA solutions caused a shift of fluorescence maximum to longer wavelengths for both excitation wavelengths and the increase of quantum yield, which was higher for λ_{exc} = 365 nm.

Upon adding EggA to the SA solution, only small changes of fluorescence intensity were observed. The fluorescence spectra of SA + EggA samples, excited at 333 nm and 365 nm (data not shown) were practically the same as those of SA (λ^{f}_{max} was not changed), and their intensity changed only slightly.

SA Bivalent Anion (Form III) and Undissociated Form of SA (Form I) in the Presence of Proteins

Fluorescence spectra of the SA + EggA and SA + BSA solution, excited at λ_{exc} = 333 nm (pH 10.5) are not shown. Only very small changes in their intensity can be observed. As can be seen, an addition of protein causes neither the shift of fluorescence band nor the change in its intensity, which is supported by quantum yield. A slightly higher value of quantum yield of SA + EggA (and + BSA) solutions for λ_{exc} = 333 nm, at pH 10.5, resulted from (small amount) of the form II at this pH, which has a higher quantum yield (Fig. 2).

A slight increase in the fluorescence intensity at pH 2 is shown in Fig. 5. This was supported by quantum yield increase in the case of EggA. This effect was the result of ligand-protein interaction leading to the complex formation. Increase in the fluorescence intensity was not observed (data not shown) in solutions of SA + BSA.

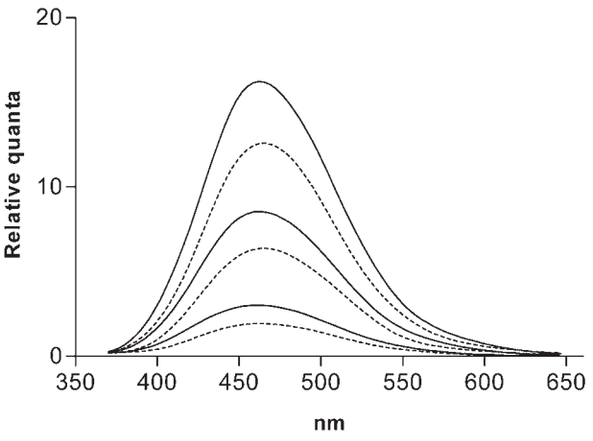


Fig. 5. Fluorescence spectra of sinapic acid–egg albumin solutions (pH 2.0) with different concentration of SA excited at 333 nm. Broken lines indicate sinapic acid solutions without protein.

Complex Parameters

The analysis was carried out for form II, SA + BSA complex at pH 6.4. The experimental results of binding are usually presented on the graph in the form of binding isotherms. Q_b was determined by extrapolation of the data presented in a coordinate system 1/Q and 1/[P_0]. The parameter *f* was determined from Eq. (7). The values of the total number of binding sites (N), binding constant (K), and (j) were calculated from binding isotherm (Fig. 6), Scatchard's graph (Fig. 7), and semilogarithmic Klotz's

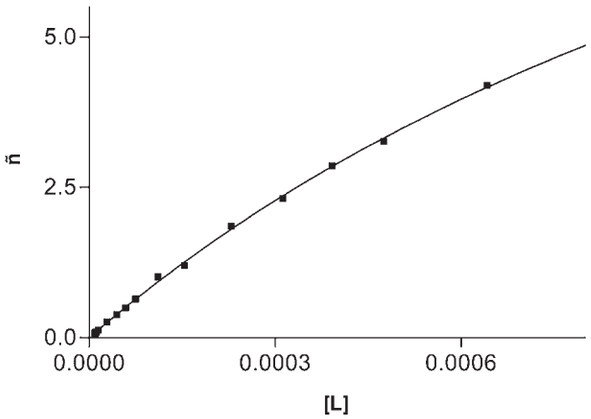


Fig. 6. Binding isotherm. ñ, An average number of bound ligand per 1 molecule of protein; [L], concentration of unbound ligand.

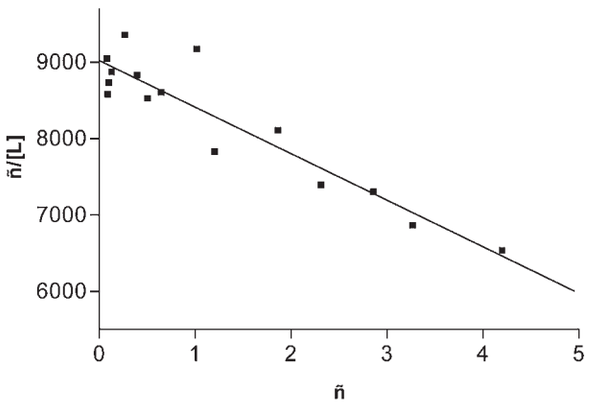


Fig. 7. Scatchard's plot. ñ, An average number of bound ligand per 1 molecule of protein; [L], concentration of unbound ligand.

plot (Fig. 8), respectively. The calculated values are presented in Table II.

Because a large degree of saturation of protein binding sites was not achieved, it is difficult to say whether there is only one type of binding site. Although the data indicate that deviation from linearity does not occur, Fig. 8 shows the degree of protein binding site saturation. This plot indicates that half of the saturation, that is, $\tilde{n} = N/2$ is not achieved.

The Hill's coefficient (j) equals 0.97, indicating the case of a simple binding. Thus, following Scatchard's graph, the effect of covering or uncovering sites because of binding (cooperation) does not occur. In the region of higher concentrations of SA, data can be treated as an extrapolation of obtained results.

Presented results, with insufficient saturation, suggest but not conclude that a monovalent anion of sinapic acid forms a complex with bovine serum albumin. The equilibrium constant for this complex is about 600 M^{-1}, there are at least 15 binding sites of one type, and this is a case of a simple binding in the region of concentration used.

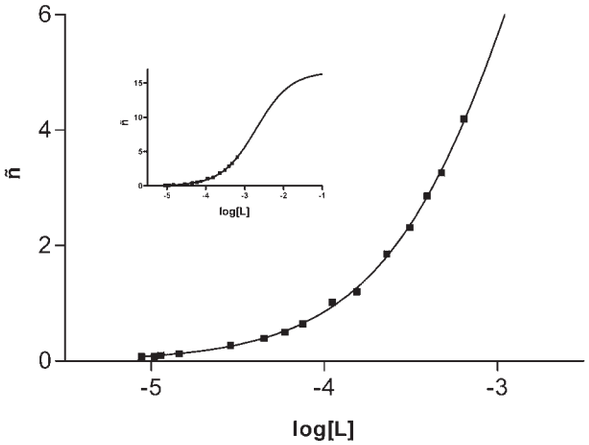


Fig. 8. Semilogarithmic plot. ñ, An average number of bound ligand per 1 molecule of protein; [L], concentration of unbound ligand.

DISCUSSION

The ratio of SA dissociation constants for OH and COOH is about $5.4 \cdot 10^4$; thus dissociation processes of these groups can be treated independently [22]. By introducing the average of dissociating protons (s), it can be proven that if s = l, then $-\log(K^c_a \cdot K^c_b) = 2 \cdot pH$ [22], where K^c_a and K^c_b are dissociation constants for COOH and OH group in the ground state. The values of pK_a in the excited state indicate that acidic character of COOH and OH groups remain the same, as it was in the ground state.

Two forms of three SA forms interact with BSA: form II of SA (a monovalent anion, pH 6.4) and form III of SA (a bivalent anion with dissociated OH and COOH groups at pH 6.4). At pH 10.5, they do not participate in such interactions. The changes in fluorescence spectra and the increase in quantum yield of form II in the presence of BSA, at pH 6.4 in relation to those of free form II, indicate that interaction occurs. BSA causes the shift of SA fluorescence spectrum of about 30 nm for $\lambda_{exc} = 333$ nm and about 50 nm for excitation $\lambda_{exc} = 365$ nm toward longer wavelengths (Figs. 3 and 4). These shifts are accompanied by a considerable increase in fluorescence quantum yield, which is higher for $\lambda_{exc} = 365$ nm. Although at pH 6.4 the amount of a bivalent anion (form III) is only 0.2% (Table I), its participation in light absorption at 365 nm is significant because of the very high extinction coefficient. As a result, the ratio of quanta absorbed by molecules with dissociated COOH groups (form II) to quanta absorbed by the molecules with additionally dissociated OH group (form III) for this wavelength equals only about 3. Simultaneously, the same values of pK_a in the ground [1] and excited states rule out the possibility of an equilibrium shift between forms II and III in the excited state. The participation of form III in the fluorescence of SA + BSA solutions at pH 6.4 is indicated also by a higher value of fluorescence quantum yield for $\lambda_{exc} = 365$ nm than that for $\lambda_{exc} = 333$ nm, and the position of λ^f_{max}, which is shifted toward longer wavelength for longer excitation wavelength (Fig. 3 and 4). For similar

Table II. Parameters of the Complex of SA (Form II) and BSA

SA + BSA	N	K [M^{-1}]	R^2
Binding isotherm	15.2 ± 1.1(7%)	590 ± 53(9%)	0.9992
Scatchard's plot	14.8 ± 2.1(13%)	610 ± 67(11%)	0.8654
Semilogarithmic plot	16.6 ± 7.2(43%)	504 ± 131(26%)	0.9992
Hill's coefficient (j)	0.97 ± 0.05		

Note: $\lambda_{exc} = 333$ nm, pH 6.4. N, Total number of binding sites; K, binding constant.

reasons the participation of a form with undissociated OH and COOH groups (form I) at this pH can be neglected.

The fluorescence spectra of SA+BSA solutions (pH 6.4) excited at 333 and 365 nm are thus complex spectra of free and bound SA forms. The fluorescence of SA free form II gives the contribution to a fluorescence spectrum excited at 333 nm and to the fluorescence spectrum of free form III excited at 365 nm. In the SA + BSA solutions, the participation of the fluorescence of free form III in spectra, at 333 nm excitation, is not big enough to explain the observed spectral shift toward longer wavelengths with respect to the fluorescence of free and bound form III. The bound SA forms include, first of all, the complex of SA II form with protein, revealing itself in the fluorescence spectrum ($\lambda_{exc} = 333$ nm) by a bathochromic shift λ^{f}_{max} and the increase in quantum yield.

The SA+BSA fluorescence spectrum excited at $\lambda_{exc} = 333$ nm was decomposed into the spectrum of free and bound form II. To achieve this, all spectra in relative quanta units were used and corrected for reabsorption. Next, the spectrum of the form II was multiplied by mole fraction $[L]/[L_0]$ and was subtracted from the spectrum obtained in the presence of protein. In this way the spectrum of the complex was obtained. The maximum of fluorescence was centered at about 470 nm. Further bathochromic shift of the spectrum ($\lambda_{exc} = 365$ nm) indicates the possibility that bivalent anion (form III), forms a complex with BSA.

The results suggest that both dissociated COOH group (form II) and dissociated OH group (form III) participate in the interaction of sinapic acid with BSA. It is confirmed by a higher quantum yield of SA + BSA solutions obtained for longer wavelength excitation, which supports the idea of more effective complex formation in the case of bivalent anions.

Another situation was observed at pH 2, where SA is present with undissociated OH and COOH groups. The fluorescence spectra of SA form I in the presence of BSA excited at $\lambda_{exc} = 333$ and 365 nm had the same position of λ^{f}_{max} as that of the free form, and the quantum yield was only slightly higher.

On the other hand, the situation in solutions at pH 10.5 is quite clear. No changes were observed in the absorption and fluorescence spectra of SA form III and BSA. The complex formation at this pH was not detected. It should be emphasized that the use of carbonate-sodium bicarbonate buffer solution with a greater ionic strength than Britton-Robinson's buffer (about fourfold) can deter the formation of the SA complex with BSA at pH 10.5 in comparison to solutions at pH 6.4 and 2. It is known that solute ions compete with ligands for the binding sites on protein [23–25], and as a result, a lower number of protein binding sites can be available in a buffer of high ionic strength.

A different situation occurred in sinapic acid solutions with egg albumin. Small changes in fluorescence spectra and quantum yield at pH 2 (form I) indicate weak interaction. Lack of these changes for forms II and III (pH 6.4 and 10.5) indicate that they do not complex with EggA.

Suggested Mechanisms of Complex Formation

The EggA structure is more rigid than the BSA structure, and its surface is more hydrated [26]. Hence, binding sites can be unattainable for sinapic acid forms. Such a structure and microenvironment match the ionization properties of the residue groups. Higher temperatures lead to the partial denaturation of EggA (probably because of the destruction of the water layer), which also causes much more effective binding of ligands—the probes of protein hydrophobic sites [27].

As far as the mechanism of SA–BSA interaction at pH 6.4 is concerned, electrostatic interaction occurs between an ionized carboxyl group (form II) and basic groups of protein residues of such amino acids as lysine, arginine, or histidine, located on the protein surface. pK_a of imidazole groups, $\varepsilon\text{-}(NH_2)^+$, $\varepsilon\text{-}(NH_3)^+$ of these amino-acids in BSA is so high that at pH 6.4 the residues of lysine and arginine are completely undissociated [11] and of histidine is partially ($pK_a = 6.9$) dissociated. An additional argument for the electrostatic interaction is that if lateral groups of arginine or lysine in BSA are blocked by chemical reactions, it causes the loss of charge and a subsequent sudden decrease in anion binding [23]. Hence, binding depends on local charge distribution on the protein. These local places can be treated eventually as specific binding sites for SA, which may be shielded by the water layer. The above findings are contrary to the hydrophobic binding mechanism proposed by Beavis [2]. The high number of binding sites (Table II) suggests that protein surface is "pasted" with SA molecules. The overall number of binding sites for other compounds on BSA can exceed 40 [11], depending on the structure of the molecule and the composition of the solution. However, only one of them can be distinguished as a strongly binding site [11,28,29].

The occupation of a large number of binding sites is possible in the case of SA at pH 6.4, especially because the majority of polar residues are exposed on the protein surface, and the number of groups with positively charged nitrogen atom is 99 (17-His, 23-Arg, and 59-Lys) [29].

The increase in pH over 10–12 for the solutions containing BSA-ligand complexes causes the decomposition

of binding. It is connected first of all with high average negative charge of BSA and the dissociation of groups ε-$(NH_3)^+$ of lysine and partially ε-$(NH_2)^+$ of arginine, which prevents lysine groups from participating in binding. The charge on the protein surface at pH 10.5 is -55 [11], and at pH 6.4 is -8 [9]. As a result, strong electrostatic repulsion exists between anions and protein, which in the case of SA (form III, a bivalent anion) prevents the process of complex formation.

At pH 2 the native structure of the protein is denaturated. pH for the transition N↔F is 3.8–4.3 for BSA and 4.0 for EggA [11]. At this pH, OH and COOH groups (form I) of sinapic acid are not dissociated. Therefore these groups can form hydrogen bonds with atoms of protein lateral groups or with the peptide chain. It is also possible that the SA aromatic ring can form van der Waals binding with lateral aromatic amino acids.

A constant and higher value of quantum yield in the presence of both proteins in relation to free SA, as well as the increase in its concentration, suggest that there are numerous binding sites for formed complex.

CONCLUSION

Presented results, without saturation data, suggest but do not conclude that in water solutions we should pay attention to the pH of the solution that can cause the changes of absorption maximum and interaction with proteins. Bovine serum albumin forms complexes with sinapic acid forms II and III at pH 6.4 by electrostatic forces rather than by hydrophobic interactions. EggA does not interact with sinapic acid at pH 6.4 and 10.5 because of the water layer surrounding the protein and binding sites. Therefore proteins similar to egg albumin may interact with SA after drying and freezing.

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