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pH-Dependent Complexation of Histamine H_1 Receptor Antagonists and Human Serum Albumin Studied by UV Resonance Raman Spectroscopy

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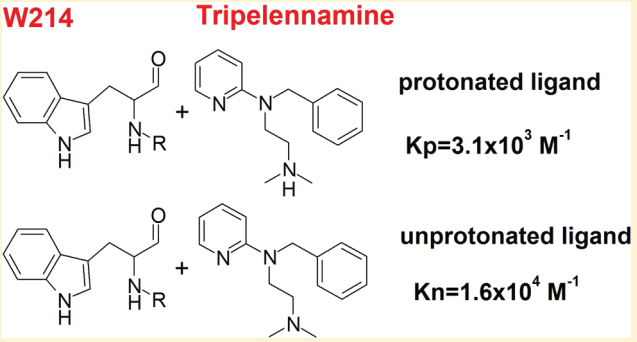
Supporting Information

ABSTRACT: UV resonance Raman spectroscopy was used to characterize the binding of three first-generation histamine H_1 receptor antagonists—tripelennamine (TRP), mepyramine (MEP), and brompheniramine (BPA)—to human serum albumin (HSA) at pH 7.2 and pH 9.0. Binding constants differ at these pH values, which can be ascribed to the different extent of protonation of the ethylamino side chain of the ligands. We have recently shown [Tardioli et al. *J. Raman Spectrosc.* **2011**, *42*, 1016−1024] that for the solution conformation of TRP and MEP the side chain plays an important role by allowing an internal hydrogen bond with the aminopyridine nitrogen in TRP and MEP. Results presented in this paper suggest that the existence of such molecular structures has serious biological significance on the binding affinity of those ligands to HSA. At pH 7.2, only the stretched conformers of protonated TRP and MEP bind in HSA binding site I. Using UV absorption data, we derived binding constants for the neutral and protonated forms of TRP to HSA. The neutral species seems to be conjugated to a positive group of the protein, affecting both the tryptophan W214 and some of the tyrosine (Y) vibrations. BPA, for which the structure with an intramolecular hydrogen bonded side chain is not possible, is H bound to the indole ring nitrogen of W214, of which the side chain rotates over a certain angle to accommodate the drug in site I. We propose that the protonated BPA is also bound in site I, where the Y150 residue stabilizes the presence of this compound in the binding pocket. No spectroscopic evidence was found for conformational changes of the protein affecting the spectroscopic properties of W and Y in this pH range.



INTRODUCTION

Histamine H_1 receptor antagonists (commonly referred to as antihistamines) are particularly effective in the treatment of allergies by interacting with the H_1 receptor. The sedating effect of the first-generation H_1 antihistamines has been associated with their ability to penetrate the central nervous system, which is likely related to weak serum protein binding.[1] Binding of those antihistamines to plasma proteins is therefore an important issue for their biological efficacy since it modulates drug availability to the intended target. In the present paper, attention is focused on the binding of antihistamines to human serum albumin (HSA), the most abundant transport protein in blood.

In a previous paper,[2] we selected two antihistamines, tripelennamine (TRP) and mepyramine (MEP), as representative, for their optical properties. As many antihistamines of that class, and as indicated in Figure 1, they share as a common feature an ethylamino chain with a strongly basic ($pK_a \approx 9$) nitrogen atom at the end. Under physiological conditions, the tail is therefore protonated. Furthermore, both TRP and MEP have an aminopyridine moiety, which is largely responsible for their chromophoric properties. Both chromophore nitrogens are unprotonated under physiological conditions: the second pK_a value for these molecules is approximately 4.[3] For comparison, another antihistamine, brompheniramine (BPA), is used, which has an ethylamino tail similar to TRP and MEP, but the chromophoric properties are rather different: the aminopyridine moiety is replaced by a pyridine, with a pK_a of approximately 5.2 also unprotonated under physiological conditions, and furthermore the central atom is an optically active carbon, instead of a nitrogen.

HSA has long been at the center of attention of the pharmaceutical industry due to its ability to bind various drugs and alter their pharmacokinetic properties. HSA is a globular protein with two principal binding sites for a wide variety of ligands located in hydrophobic cavities in subdomains IIA (site I) and IIIA (site II). A single tryptophan residue in HSA (W214) is located in binding site I.[4]

Several papers report methodologies on separations to determine the association equilibrium constants for binding

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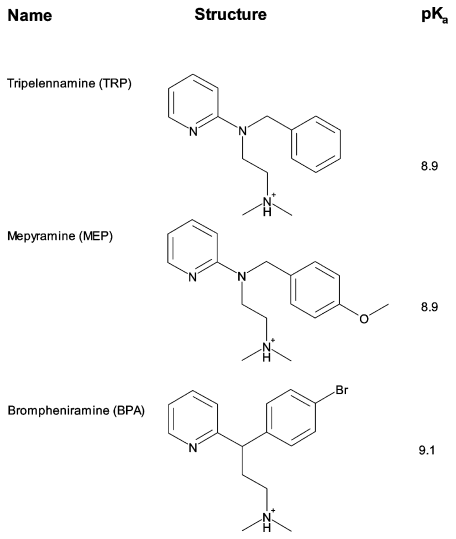

Figure 1. Structures of the antihistamines under physiological conditions. The pK_a value given is for the ethylamino side chain nitrogen. The other nitrogens are unprotonated under physiological conditions.

of these antihistamines to proteins,[5−7] but the elucidation of the binding mechanism has hardly been attended. For this purpose, vibrational spectroscopic techniques, which provide detailed structural information, could be invoked. In particular, UV resonance Raman (UVRR) spectroscopy can in principle provide detailed data on how a ligand is bound. In view of this, complex formation between HSA and the three antihistamines was investigated in aqueous solution at two pH values 7.2 an 9.0, by means of UVRR spectroscopy. These pH values were chosen to elucidate the influence of the protonation of the ethylamino tail on the binding properties. Of course, one should know also whether the protein structure is affected in this region. Although conformational changes were reported for HSA in binding experiments as a consequence of pH change,[8] in the pH range between 7 and 9 so far no changes in the optical properties of the tryptophans such as absorption or fluorescence spectra have been observed.[9,10] We confirm those findings by showing in this paper that no changes in the UV absorption or in the UVRR spectra are observed either.

UVRR spectroscopy is performed at 244 nm excitation, a laser line provided by the continuous wave frequency doubled argon ion laser. This is a suitable wavelength, not only because it is in resonance with the amino pyridine moiety of TRP and MEP and in between the $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transitions of the pyridine part of BPA but also because it is in resonance with the tryptophan and tyrosines of HSA, so that in principle difference spectra can give information about possible complexation induced changes in these amino acids as well. We recently made use of the same technique to successfully investigate the solution conformation of TRP and MEP.[2] We showed that there is a definite influence of the protonated ethylamino chain on the chemical and photophysical properties of these molecules. Most probably for TRP and MEP the HSA−ligand interaction is affected by their solution conformations, which in turn are dependent on the protonation state of the tail. It was found that the protonation state of the tail influences the chromophoric properties as well as the pK_a values of the aminopyridine moiety. Compared to isolated aminopyridines, K_a values are 2 orders of magnitude lower in TRP and MEP. We concluded that in addition to the normal stretched conformer also a bent conformer plays a role. The bent conformation allows interaction of the tail nitrogen with the chromophore, by either formation of a hydrogen bond or direct interaction with the ring π-electrons. These findings explain why Ter Laak and co-workers[1] in a previous study of the binding of H_1 antihistamine to serum proteins by equilibrium dialysis coupled to UV detection found that the ionization state of MEP strongly influences its binding to serum proteins in the pH range from 6.0 to 9.0. The differences in MEP binding at different pH values indicate different affinities of the ionized and the unionized form of MEP for HSA.[1]

The aim of the present study is to obtain more detailed structural information about the interaction of TRP and MEP with HSA. The bent conformer only exists when the tail is protonated, and upon deprotonation only the stretched conformer is expected. For BPA such complications are not encountered; therefore, it is quite suitable for comparison.

EXPERIMENTAL SECTION

Materials. Essentially fatty acid free human serum albumin (HSA), tripelennamine hydrochloride (TRP), mepyramine maleate (MEP), and brompheniramine maleate (BPA) were purchased from Sigma Aldrich and used without further purification. Binding experiments were performed at pH 7.2 and pH 9.0 by dissolving the protein and the antihistamine salts, respectively, in 20 mM phosphate buffer (sodium monophosphate−sodium diphosphate) and 20 mM borate buffer (boric acid−sodium hydroxide). Water was purified by passage through a Millipore Milli-Q system.

Sample Preparation. UV titration measurements of HSA were performed in the presence of TRP and MEP at pH 7.2 and pH 9.0. The protein concentration was kept constant at 120 μM, while ligand concentration was varied between 0 and 600 μM. The range was chosen in accordance with the values of the known association constants of the antihistamine salts to HSA.[3] The binding of BPA to HSA was not investigated by UV titration since under physiological conditions the ligand has a very low affinity ($K_a = 1.8 \times 10^3$ M^{-1}) for the protein.[5]

UV absorption spectra of the TRP and MEP in the absence and in the presence of the protein were recorded at room temperature using a 0.5 mm quartz cell. In the range investigated, no concentration effects on the UV absorption spectra of the antihistamine salts were observed. Self-association of the compounds studied starts to be important in aqueous solution at much higher concentrations than used in this study.[11] For the binding experiments, 120 μM HSA buffer solution was prepared and partly used to dissolve TRP and MEP at a concentration of 1.03 and 1.05 mM, respectively. Different aliquots of the ligand/protein solution were added to the protein solution so that a constant protein concentration was maintained throughout the titration. To record the spectra of the free ligands at the same concentrations as used in the titration, TRP and MEP were also dissolved in buffer (without the protein) at a concentration of 1.03 and 1.05 mM, respectively. The same volumes of TRP and MEP used in the binding experiment with HSA were added to the buffer solution.

UVRR spectra were measured at a protein concentration of 120 μM and ligand concentrations of 500 μM at pH 7.2 and of 250 μM at pH 9.0, for both TRP and MEP. In virtue of the low K_a of BPA to HSA, the concentration used for this compound was 1.13 mM at both pH 7.2 and pH 9.0. Na_2SO_4 (50 mM)

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was added to all samples as internal standard for Raman intensity measurements. $NaClO_4$ could not be used: it was found to aggregate with the ligands.

Instrumentation. UV electronic absorption spectra were measured on a Varian Cary 50 Bio UV−Vis spectrophotometer. Continuous-wave UV radiation of wavelength at 244 nm was obtained from an intracavity frequency-doubled argon ion laser, Innova FreD 90C (Coherent, Utrecht, The Netherlands). The UVRR setup was described in detail previously.[12]

UVRR Detection System. Raman scattering was collected from a vertically dripping flow of sample solution (6 mL) delivered by a peristaltic pump (Gilson Minipuls 2) coupled to a peek tube with a flow rate of 100 cc/min. Thus, measurements were done directly on a free hanging droplet. Droplet size can be controlled by adjusting the flow rate and was optimized for the focal length of the objective. The setup was specifically designed for this experiment to enable detection of the Raman scattered light from the protein solution. Previous measurements utilizing a flow through a horizontally mounted quartz capillary showed that HSA stuck to the wall cell at the spot where the laser light was focused, thus seriously hindering the Raman signal from the protein solution circulating in the capillary. The present drop-flow system gave outstanding results in terms of sensitivity even at short times of collection. A 3600 lpm line grating was used to disperse the spectra on a cooled (−50 °C) Andor technology model DV420-OE CCD camera (Belfast, Northern Ireland) in the range 500−2000 cm^{-1}. The S/N ratio was improved by repetitive scanning (45 times) during 10 s for all samples. No time-dependent changes were observed from scan to scan, indicating the stability of the dripping sample in the UV laser beam. Also, UV absorption spectra taken before and after the RR data acquisition showed no evidence of laser-induced photodegradation for any of the compounds investigated.

UVRR spectra of HSA, BPA, and BPA/HSA solution at pH 7 and 9 first had the blank subtracted, by scaling intensities using the 981 cm^{-1} band of SO_4^{-2}, to remove a weak Raman contribution of the H_2O bending mode around 1640 cm^{-1} and to remove the curvature of the baseline due to the sensitivity of the spectrometer. After scaling and the blank subtraction, difference UVRR spectra were determined. In the UVRR spectra of BPA, HSA, and BPA/HSA shown in Figure 4, the salt band at 981 cm^{-1} is absent because of the required blank subtraction. For TRP and MEP, scaling with the 981 cm^{-1} band of SO_4^{-2} was not immediate. In fact, both TRP and MEP show resonance enhancement at 984 cm^{-1} and the band overlaps with the sulfate calibration band at 981 cm^{-1}. In those cases, UVRR spectra of the ligands in the presence and in the absence of the salt were recorded, and by subtraction of the two spectra the salt spectrum was evaluated. The relative intensity of the ligand band at 984 cm^{-1} (in the absence of the salt) with respect to the derived salt spectrum was used to scale the UVRR spectrum of the ligand band (in the presence of the salt), composed by the salt band and TRP band. After scaling, again the blank spectrum was subtracted to remove the weak Raman contribution of the H_2O bending mode at 1640 cm^{-1} and to remove the curvature of the baseline due to the sensitivity of the spectrometer. The same scaling procedure was adopted for the TRP/HSA complex and MEP/HSA complex after recording the UVRR spectra in the presence and in the absence of the salt.

Difference Spectra. The pH dependencies of the UV absorption and UVRR spectra of the antihistamines investigated were determined by subtraction of the spectrum at pH 7.2 from the spectrum at pH 9.0 at equimolar concentrations. For each antihistamine investigated in this study, the changes in spectral properties induced by complexation between the investigated antihistamines and HSA were evaluated at one point of the titration by subtracting the free ligand spectrum (no HSA) and the free HSA spectrum (no ligand) from the recorded spectrum of the complex. Such difference spectra originate from those molecular groups affected by binding. Since all passive groups are invisible, the difference spectrum exhibits details of the binding mechanism.

RESULTS AND DISCUSSION

UV Absorption Spectra. To study the influence of protonation on the ligand structure and their binding to HSA by UVRR spectroscopy, UV absorption spectra should be recorded since pH changes might affect the molar extinction coefficients and thus the resonance enhancement effect in UVRR at 244 nm. First, the influence on the antihistamine spectra will be discussed and subsequently the ligand−HSA combinations.

Antihistamines. In Figure 2 the UV absorption spectra of TRP and BPA are presented at two pH values. For both ligands

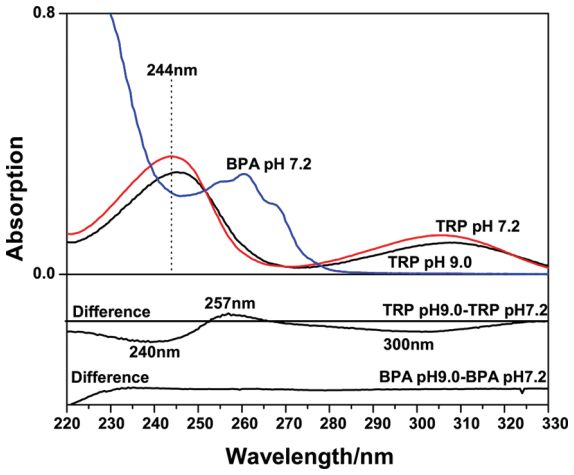

Figure 2. UV Absorption spectra of: TRP (500 μM) (red line, pH 7.2; black line, pH 9.0) and BPA (1.13 mM) (blue line, pH 7.2). The associated difference spectra (pH 9.0−pH 7.2) are also shown. 244 nm is the emission wavelength of the FreD laser used to record UVRR spectra.

the difference spectra associated with the pH change from 7.2 to 9.0 are included as well. The UV spectral profile of MEP (not shown) resembles that of TRP, though additionally rather weak absorption bands from the anisole chromophore are observed below 230 and at 275 nm, which are not influenced by the pH change.[3]

The UV absorption spectrum of BPA shown in Figure 2 has close spectroscopic resemblance to the absorption spectrum of the pyridine molecule.[13] The difference spectrum is a flat horizontal line: its chromophoric properties are not affected by a change in pH, consistent with the idea that there is no interaction between the protonated ethylamino tail and the pyridine chromophore. The negative feature below 230 nm is related to the acid−base equilibrium of the maleate counter ion.

TRP and MEP behave quite differently from BPA; their difference spectra exhibit several positive and negative features,

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indicating band shifts. At pH 7.2, TRP and MEP exhibit similar bands centered at 245 and 306 nm. These are ascribed to electronic transitions within the aminopyridine chromophore at 245 nm and to a charge transfer transition from the amino substituent to the π-ring at 306 nm, which are conjugated.[2,3] At pH 9.0 a wavelength shift of the two bands is recorded, resulting in a difference spectrum with negative features at 240 and 300 nm and a positive component at 257 nm. These wavelength shifts are thought to be caused by a difference in interaction between the tail and the amino pyridine moiety at pH 7.2 and 9.0. This interaction is significant in the bent conformation if the tail is protonated and could be hydrogen bond formation or direct interaction with the pryidine π-electrons. At pH 9 only 50% of the molecules will be protonated at the tail (pK_a = 8.9).[2,3]

Antihistamine/HSA Complex and K_a Evaluation. The pH influence on the binding of TRP and MEP to HSA was first investigated by means of UV absorption spectroscopy. The UV absorption spectra of HSA, TRP, and the TRP/HSA complex are presented at pH 7.2 in Figure 3. Absorption spectra

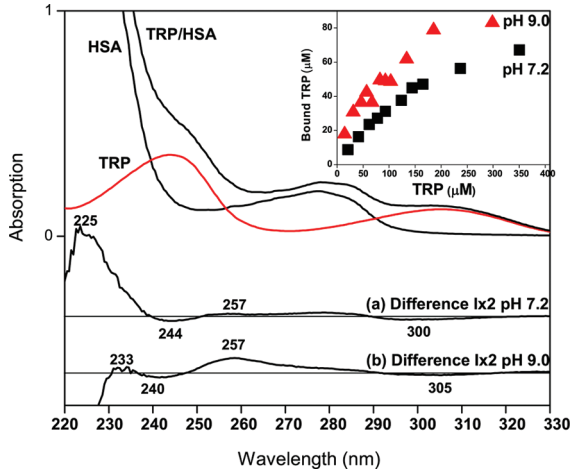

Figure 3. UV absorption spectra of HSA, TRP, and the TRP/HSA complex at pH 7.2. (a) Difference spectrum (TRP/HSA−HSA−TRP) at pH 7.2; (b) at pH 9.0. In the inset, the dependence of protein-bound TRP vs [TRP] measured at 255 nm is shown at pH 7.2 (black squares) and pH 9.0 (red triangles).

evaluated at pH 9.0 are not included. Emphasis is on the difference spectra (TRP/HSA−HSA−TRP) at pH 7.2 (a) and pH 9.0 (b). Only those features whose intensity increases upon TRP addition are labeled. We emphasize that no change in the shape and intensity of the HSA absorption spectrum itself was found when going from pH 7.2 to pH 9.0, indicating that in this pH range no conformational change influencing the absorption of the protein takes place.

In the difference spectrum at pH 7.2, complex formation induces negative features around 244 and 300 nm and a weak positive band at 257 nm, all ascribed to the ligand. This is quite similar to the difference spectrum shown in Figure 2, showing the effect of pH on the structure of TRP as in the absence of HSA. This similarity in difference spectra strongly suggests that upon complexation with HSA at pH 7.2 the stretched configuration of TRP is favored, even though in solution at that pH this conformation is present in only a minor amount. This finding is in line with the UVRR data of the TRP/HSA complex at pH 7.2. Results, as described later in the paper, indicate that the pyridine nitrogen N of TRP is involved in binding with the NH group of tryptophan indole, with a concomitant increase in hydrophobicity of the environment. Therefore, only the stretched conformer of TRP, i.e., the one with an AP chromophore not involved in intramolecular H-bond, is able to bind in site 1 of HSA.

In the difference spectrum at pH 7.2, another weak band is observed around 275 nm. This band does not exist in the difference spectrum in the absence of HSA, and it is therefore ascribed to the absorption of the only tryptophan amino acid residue within the protein, taking place at around 280 nm in the absence of the ligand. The indole NH residue is thought to interact with the pyridine nitrogen of TRP, as suggested by UVRR results. The blue-shift may reflect the change to a more hydrophobic environment experienced from the residue upon binding.

The difference spectrum at pH 9.0 is quite similar in shape to that at pH 7.2, apart from the region below 240 nm, although the peaks appear to be slightly shifted and the peak at 257 nm is more pronounced. When the concentration of bound TRP is plotted as a function of the TRP concentration (see inset of Figure 3), there is an initial linear change up to about 120 μM (the HSA concentration in these experiments), with a steeper slope at pH 9.0 than at pH 7.2. These results indicate the formation of a 1:1 complex. The association constant calculated at pH 9.0 is 9.5×10^3 M^{-1}, more than three times larger than the value at pH 7.2 of 3.1×10^3 M^{-1}. The model and evaluation of the binding constants are available in the Supporting Information. This implies that the binding constant of the neutral component, present at about 50% in solution, is about five times larger than that of the protonated form (1.6×10^4 M^{-1}). This in turn implies that—if the difference in binding constants is attributed to the difference in protonation state—about 80% of the HSA-bound drug is in its neutral form at pH 9.0.

The difference spectra at pH 9.0 and pH 7.2 are markedly different in the wavelength region below 240 nm. Whereas at pH 7.2 the 225 nm band is positive, it is negative at pH 9.0. At pH 7.2 complex formation with TRP gives rise to a blue-shift in the HSA spectrum but at pH 9 to a red-shift. The interpretation is not straightforward since in this region a number of effects can contribute to the difference spectrum. The observed absorption can not be fully ascribed to the Bb (π−π*) band of tryptophan (W214) in HSA, as also the Lb (π−π*) transitions of the tyrosine residues contribute to the intensity. Nevertheless, the results indicate that complexation of HSA with positively protonated TRP gives rise to another spectrum than complexation with neutral TRP. Presumably, complexation with a protonated TRP induces a shift of the W214 band to the red, in line with the observation that interaction with a proton at the NH side of the indole ring causes a red-shift in a hydrophobic environment and an intensity increase of the Bb absorption,[14−16] whereas complexation of HSA with the neutral form causes the shift of the spectrum to the blue.

UVRR Spectra. UVRR spectra of all the compounds investigated and their complex with HSA are presented and discussed in this section. Excitation was performed at 244 nm, suitable for enhancement of both the protein and the ligands. The concentrations of HSA (120 μM) and the ligands (500 and 250 μM) gave rise to similar signal-to-noise ratios.

The 244 nm excitation wavelength favors in HSA the resonance enhancement of the vibrations of tryptophan, tyrosine, and, to a minor extent, the peptide backbone.[15,17,18]

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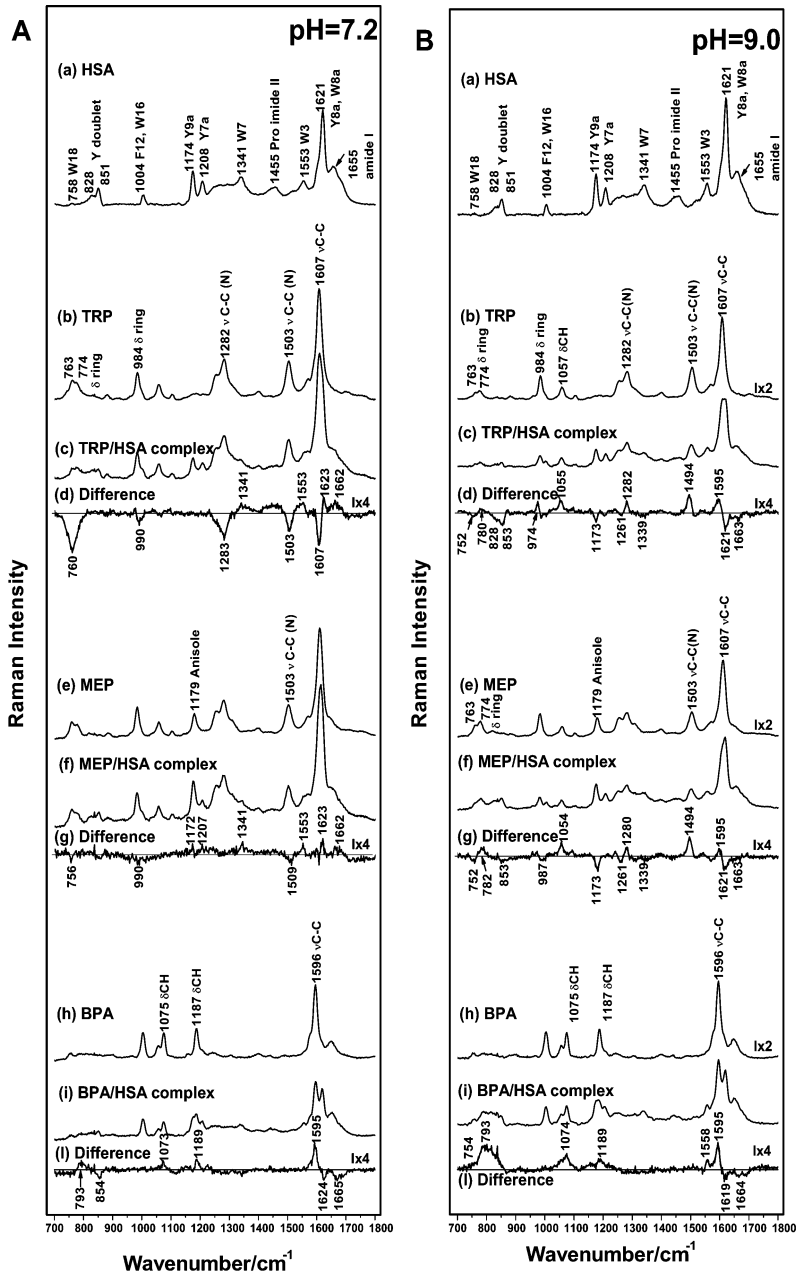

Figure 4. A: UVRR spectra at pH 7.2, 244 nm excitation: (a) HSA 120 μM; (b) TRP 500 μM; (c) TRP/HSA complex; and (d) difference (c) − ((a) + (b)); (e) MEP 500 μM, (f) MEP/HSA complex; and (g) difference (f) − ((a) + (e)); (h) BPA 1.13 mM; (i) BPA/HSA complex; and (l) difference (i) − ((a) + (h)). B: UVRR spectra at pH 9.0, 244 nm excitation: (a) HSA 120 μM; (b) TRP 250 μM; (c) TRP/HSA complex; and (d) difference (c) − ((a) + (b)); (e) MEP 250 μM, (f) MEP/HSA complex, and (g) difference (f) − ((a) + (e)); (h) free BPA 1.13 mM; (i) BPA/HSA complex; and (l) difference (i) − ((a) + (h)).

Also, the 244 nm excitation is in resonance with the aminopyridine moiety of TRP and MEP and with the pyridine chromophore of BPA. Due to the overlap of the UVRR spectra of the antihistamines and HSA, use was made of difference spectra to distinguish between spectral changes originating from HSA and those arising from the ligands. In Figure 4 we show the UVRR spectra at pH 7.2 and pH 9.0 of HSA, antihistamine, and the antihistamine/HSA complex and the associated difference spectra for the three ligands. In Figure S1 of the Supporting Information we provide a description of the UVRR spectra of TRP, MEP, and BPA and the difference spectra related to changes in pH and polarity, with the complete assignments done according to refs 2 and 19. In Figure 4, only those ligand vibrations modified by binding are labeled.

HSA. The UVRR spectra of HSA at pH 7.2 and pH 9.0 are displayed in the top traces of Figure 4A and Figure 4B, respectively. At 244 nm excitation, strong enhancement is observed for the Raman bands arising from the single tryptophan, 18 tyrosine, and 31 phenylalanine residues,[20] in preresonance through their Bb (tryptophan) and Lb (tyrosine) electronic transitions. No modification of the UVRR profile of HSA was detected with pH change, thus confirming previous results from Harada and co-workers,[15] who first reported the UVRR of HSA. This is in line with the observation that no appreciable changes are found in the optical properties of HSA

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Table 1. Vibration Band Assignments of HSA[15]

human serum albumin		
band (cm^{-1})	assignment	description
758	W18	symmetric benzene/pyrrole in-phase breathing
828	2Y16a	out-of-plane ring deformation
851	Y1	symmetric phenol in-phase breathing
1004	W16	symmetric benzene/pyrrole out-of-phase breathing
1174	Y9a	in-plane CH bend, C_6H_5-C
1208	Y7a	ring−C stretch
1341	W7	pyrrole ring vibration
1455	Pro Imide II	Pro C−N stretch
1553	W3	C_2-C_3 pyrrole ring stretch
1621	W1, Y8a	phenyl mode with contribution from the pyrrole NC stretch
1655	Amide I	C═O stretch with minor contribution from the out-of-phase C−N stretch

upon this pH change. Observable Raman bands arising from the tryptophan (W) and tyrosine (Y) residues are listed in Table 1 and assigned using ref 15.

The UVRR bands of the single tryptophan (W214) in HSA show up at 1621 cm^{-1} (W1 overlapping with Y8a, aromatic C−C stretching), 1553 cm^{-1} (W3, C−C stretching of the pyrrole ring), 1347 cm^{-1} (W7, tryptophan doublet), 1004 cm^{-1} (W16, benzene ring breathing overlapping with F12), and 758 (W10) cm^{-1}. Those vibrations, except W1, are strongly enhanced with excitation in preresonance with the Bb electronic transition of tryptophan (~220 nm).[21]

The UVRR bands of the tyrosine residues are observed at 1621 cm^{-1} (Y8a), 1208 cm^{-1} (Y7a, ring−C stretching), and 1174 cm^{-1} (Y9a, CH in-plane bending). We also recorded the tyrosine Fermi doublet at 828/851 cm^{-1} which originates from resonance between the symmetric ring breathing Y1 and the overtone of the out-of-plane deformation mode Y16a of the ring vibration at ~413 cm^{-1}. Phenylalanine only contributes at 1004 cm^{-1} (F12, ring breathing) where it overlaps with W16 of tryptophan. Additional peptide vibrations occur at 1455 cm^{-1} (Pro C−N stretch, Amide II) and 1655 cm^{-1} (Amide I).[18,22] Those latter vibrations depend on the secondary structure of the protein.

TRP/HSA Complex. In Figure 4A in trace (c) the UVRR spectrum of the TRP/HSA complex at pH 7.2 and in trace (d) the associated difference spectrum (complex−HSA−TRP) are presented.

Upon binding, several negative features show up in the difference spectrum which can be attributed to the aminopyridine moiety of TRP: at 760 cm^{-1} δ_{ring}, at 1283 cm^{-1} and 1505 cm^{-1} $\nu_{C--C(N)}$, and at 1607 cm^{-1} ν_{C--C}. The same vibrations also show up in the pH difference spectra for TRP as such, as shown in Figure S1 (Supporting Information). They are affected by the protonated tail in the bent conformer at pH 7.2. Similar behavior was observed in the UV absorption spectra as detailed above. The lower wavenumber component of the doublet at 760/774 cm^{-1} decreases in intensity upon binding to HSA. This band originates from the ring stretching 6a of a H-bound AP moiety.[2] The intensity ratio of the 1257/1282 cm^{-1} doublet decreases upon binding, following the behavior we recorded for TRP from pH 7.2 to pH 9.0. For both 760/774 cm^{-1} and 1257/1282 cm^{-1} doublets, the reduction in intensity is consistent with the absence of a hydrogen bond with the aminopyridine moiety, as described in Figure S1 of Supporting Information. The difference spectra originate exclusively from those molecular groups affected by the binding. Thus trace (c) in Figure 4A indicates that the stretched and neutral conformer dominates upon complexation with HSA, whereas the bent conformation only plays a minor role.

Some bands originating from HSA change as well. Complexation induces in the difference spectrum a weak but significant intensity increase of the W214 bands at 1341 cm^{-1} (W7), 1553 cm^{-1} (W3), and 1621 cm^{-1} (W1). Furthermore, complex formation induces an up-shift of the amide I band, which is found at 1663 cm^{-1} in the difference spectrum, and suggests a mild structural change of the protein toward an irregular α-helix.[23] The intensity increase of W7, W3, and W1 Raman bands is likely caused by the red-shift of the Bb electronic transition, in line with the differences in UV absorption spectra discussed above. The red-shift of Bb correlates with increased H-bonding of the indole group of tryptophan in a hydrophobic environment.[24] The difference spectrum of the TRP/HSA complex at pH 7.2 therefore indicates that a protonated TRP in the stretched conformation is bound (close) to W214, located in site I.

The difference spectrum at pH 9.0 clearly differs from that at pH 7.2. In Figure 4B the UVRR spectra of the TRP/HSA complex at pH 9.0 and the associated difference spectrum (Mix−HSA−TRP) are shown in traces (c) and (d), respectively. In view of the pK_a of TRP (8.9) under this condition, about half of the molecules are unprotonated at the amino side chain, and half the TRP will be still protonated. In the difference spectrum, we note that the intensities of those bands of W214 characterizing the complex formation between protonated TRP and HSA, at 990, 1341, 1553, and 1623 cm^{-1}, which give positive signals at pH 7.2 are hardly observable anymore. Apparently at pH 9.0 complexes between the protonated form and HSA are no longer formed to any appreciable extent. It can be concluded from the larger binding constant at pH 9.0 that the binding with the neutral form of TRP is much stronger, and an analysis shows that even though in solution still half the TRP is protonated in the bound complex only about 20% of TRP will be protonated.

The difference spectrum at pH 9.0 shows other positive bands than the spectrum at pH 7.2. Those originate from TRP and are observed at 974 cm^{-1} (ring bending), 1055 cm^{-1} (CH bending, 18a), 1280 cm^{-1} (C−CN stretch, 14), 1494 cm^{-1} (C−CN stretch, 19a), and 1595 cm^{-1} (C−C stretch, 8a). Compared to the spectrum of free TRP, three vibrations ascribed to ring stretches are down-shifted: those at 974 cm^{-1} (was 984 cm^{-1}), 1494 cm^{-1} (was 1503 cm^{-1}), and 1595 cm^{-1} (was 1607 cm^{-1}). Since these are ring vibrations, it implies that the π electron cloud of the AP moiety is influenced to a certain extent by complexation. Intensities also change; the intensity

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increase of TRP vibrations is again in line with the red-shift of the absorption at 244 nm (cf. Figure 3).

Negative peaks related to HSA vibrations are observed at pH 9.0 at 828/853 cm^{-1} (Fermi doublet; Y1, Y16a), 1174 cm^{-1} (Y9a), and 1621 cm^{-1} (Y8a and W1). The change in W214 modes induced by complexation is only marginal at pH 9.0, and the main changes originate from tyrosine modes in this case. The negative feature at 1621 cm^{-1} derives mainly from the Y8a vibration, where the C−C stretch of the phenolic ring takes place, but we do not exclude a small contribution from the W1 band of W214. Both Y8a and Y9a tend to decrease in more polar solvents.[14]

MEP/HSA Complex. For MEP, the results are quite similar to those obtained for TRP. The main difference is that the signal-to-noise ratio of trace (g) is distinctly lower than that of trace (d), due to the fact that binding of MEP to HSA is not strong at pH 7.2. Therefore, we do not discuss Figure 4A, dealing with MEP binding in detail. At pH 9.0, this signal-to-noise ratio is much better. In Figure 4B trace (f), the UVRR spectrum of the MEP/HSA complex at pH 9.0 and the associated difference spectrum (g) (Mix−HSA−MEP) are presented. The strong resemblance with the difference spectrum of the TRP/HSA complex at the same pH value is quite convincing and strongly suggests that the same type of binding occurs for both TRP and MEP with HSA.

BPA/HSA Complex. For BPA the difference spectra at pH 7.2 and 9.0 are quite similar, in contrast to the spectra of TRP and MEP (see Figures 4A and 4B), indicating that there is no substantial difference between the binding of protonated and neutral BPA. Positive peaks are observed at 1074 cm^{-1} (CH and NH bending), 1189 cm^{-1} (CH bending), and 1595 cm^{-1} (C−C stretch). They are ascribed to the pyridine moiety of BPA and probably reflect a change in environment to less polar upon binding to HSA.

The difference spectrum at pH 9.0 shows an additional positive peak at 1558 cm^{-1}. It originates from tryptophan (W3), but due to binding it is shifted by some 5 cm^{-1}. According to the literature this suggests that the dihedral angle about the bond connecting the indole ring with the C-atom of the amino backbone is changed.[25]

The negative features observed upon complexation at both pH 7.2 and 9.0 originate from HSA, in particular from tyrosine residue(s): 1624 cm^{-1} (Y8a) and 1664 cm^{-1} (amide I); in addition, a weak band at 854 cm^{-1} (Y Fermi doublet) is visible. A positive band is observed at 793 cm^{-1}. Since it cannot be assigned to a distinct HSA or BPA vibration, it might point to a vibration exclusively showing up in the complex.

A detailed interpretation of the BPA−HSA results accounting for these minor differences observed at different pH values is not straightforward. Nonetheless, we note that the difference spectra for BPA at both pH values are quite similar to those of MEP and TRP at pH 9.0, as far as the HSA peaks are concerned. This applies to the negative 854 cm^{-1} and 1619−1624 cm^{-1} peaks from tyrosine and the 1664−1665 cm^{-1} amide I band.

To summarize the BPA results, the effect of complexation on W214 vibrations is marginal at pH 9.0 and absent at pH 7.2. Therefore, it is not possible to conclude whether BPA (in its protonated and neutral form) enters preferably in site I or site II. To answer this question, we investigated the complexation in a separate study of the BPA−HSA system by W214 phosphorescence: a strong quenching by BPA is observed which can be explained assuming a close proximity between BPA and W214.[26]

CONCLUSIONS

This paper represents a first attempt to characterize the interactions of three antihistamines, TRP, MEP, and BPA, with HSA by means of UV absorption and UVRR spectroscopy at 244 nm excitation. Using a novel experimental setup where spectra are taken in hanging droplets, thus avoiding coagulation of protein at the cuvette surface, we were able to obtain excellent UVRR spectra of bound complexes. Under this excitation condition, tryptophan and tyrosine residues within the protein are resonance enhanced, and the same holds for the pyridine chromophore of the three ligands, which are therefore used as intrinsic probes. The three antihistamines investigated present a wide range of behaviors with respect to their binding toward HSA, which is dependent on the protonation state of the amino side chain but for TRP and MEP also on the presence in solution of stretched and bent conformers at physiological pH.

The above results make clear that for the antihistamines TRP and MEP the structure of their complexes with HSA is strongly pH dependent. In free solution at pH 7.2 where the alkylamino tail is protonated, two ligand conformations play a role, i.e., a bent structure allowing interaction of the protonated tail with the aminopyridine moiety and a stretched conformation in which such an interaction is negligible. The UVRR spectra taken at 244 nm indicate that in the HSA complex with TRP and MEP at pH 7.2 the stretched conformer dominates, whereas the bent conformation is hardly observed. Conversely, at pH 9.0—although in solution about half the ligand molecules are still protonated—HSA primarily forms a complex with neutral stretched ligands. The protonated stretched form is not visible in the spectra. From our results we can conclude that the association constant for the neutral form is considerably larger than that for the protonated form. For MEP no accurate determination could be made at pH 7.2, but for TRP it is about five times larger for the neutral form (1.6×10^4 M^{-1}) than that for the protonated form (3.1×10^3 M^{-1}).

For BPA, an antihistamine with a structure that shows some similarity to those of TRP and MEP, such pH effects are not observed. For this molecule, also containing the alkylamino tail, the protonated form will not give rise to a bent conformation as found for TRP and MEP. This is fully in line with the fact that protonation of the tail does not influence the UV absorption spectrum. Also in UVRR the effect of protonation is only minor: presumably also for this antihistamine the stretched neutral form dominates in the complexation. This interpretation is consistent with the observation that the difference spectra are quite similar—as far as the HSA vibrations are concerned—to those of MEP and TRP at pH 9.0. For all ligands, it can be concluded that binding to HSA alters the protein secondary structure since the Amide I vibration is affected.

ASSOCIATED CONTENT

Supporting Information

The model used for the evaluation of binding constants of TRP and MEP to HSA. We also provide a description of the UVRR spectra of TRP, MEP, and BPA and the difference spectra related to changes in pH and polarity, with the complete assignments done according to refs 2 and 19. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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