Binding of Ochratoxin A Derivatives to Human Serum Albumin

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Ochratoxins are fungal metabolites known to contaminate human and animal feed. Ochratoxin A (OTA) is the most widespread form of the toxins and is believed to be responsible for human renal diseases. For the majority of its lifetime within the body, OTA remains bound to the plasma protein human serum albumin (HSA). In this paper, the binding of three OTA derivatives (ochratoxin B (OTB), ochratoxin hydroquinone (OHQ), and *O*-methylated OTA (MOA)) to HSA is examined using optical spectroscopy. The binding constants decrease as follows: OTA^{2-} (5.2×10^6 M^{-1}) > OTB^{2-} (1.8×10^6 M^{-1}) > $OHQ^{2-} \sim OHQ^-$ (2.2×10^5 M^{-1}) > MOA^- (3×10^4 M^{-1}). Studies of the binding of OTB, OHQ, and MOA to recombinant proteins corresponding to the domains of HSA reveal binding to all domains but with different affinities. Similar to OTA, all derivatives exhibit the largest binding constant for domain 2. These ligands are displaced by 2,3,5-triiodobenzoate (TIB), indicating they share a common binding site and bind to Sudlow Site I within domain 2 of HSA. Derivatives with ionizable phenolic protons exhibit a decreased pK_a by as much as two units upon interaction with HSA. The magnitude of the change in pK_a observed upon binding decreases in the order OTA > OTB > OHQ. These data suggest a model in which the monoanions of OTA, OTB, and OHQ undergo deprotonation by an arginine within domain 2 upon binding to HSA. The difference in binding constant for the three dianions studied results from the stabilization of the dianion by the surrounding protein matrix.

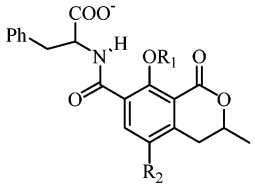
Introduction

Ochratoxins are secondary metabolites produced by several toxicogenic species of *Aspergillus* and *Penicillium* fungi.[1,2] The most abundant form, ochratoxin A (OTA), is an established human and animal food contaminate.[3−5] Concern about long-term, low dose exposure to the toxin has risen because OTA is associated with human renal diseases[6−15] and is detected in human blood samples worldwide.[6,7,13,16]

The half-life of the toxin within the body increases upon binding to blood plasma.[8,13,17−22] Binding constants have been determined for human and animal serum albumin, the predominant plasma protein.[22−24] One of the primary functions of human serum albumin (HSA) is to transport ligands throughout the body. Small ligands typically orient themselves into one or both binding cavities known as Sudlow Site I (site I) and Sudlow Site II (site II). These cavities are located in domains 2 and 3 of the protein, respectively.[25] Our previous work illustrates OTA binds to HSA in two locations with binding constants of $K_1 = 5.2 \times 10^6$ M^{-1} (domain 2) and $K_2 = 1.0 \times 10^5$ M^{-1} (domain 3).[26,27] However, the molecular details of the interactions between OTA and HSA remain unknown.

In this paper, the results of our investigation of the interaction of three derivatives of OTA with HSA are reported. The three derivatives examined are the naturally occurring dechlorinated analogue of OTA, ochratoxin B (OTB), the *O*-methyl ester of OTA (MOA), and the hydroquinone of OTA (OHQ) (see Chart 1). Structural modifications in these derivatives are localized in the aromatic moiety of the molecule. The lactone ring and amino acid side chain (Phe) are unaffected. A comparative study of HSA interaction with OTA and its derivatives should help to understand preferential binding at the molecular level. At physiological pH, the carboxylic acid present in the molecules is deprotonated (p$K_a \sim 4$).[23,24,28−30] In addition, deprotonation of the isocoumarin moiety occurs around neutral pH because the pK_a of OTA, OTB, and OHQ ranges from 7 to 8.[23,24,29,31,32] Our previous work demonstrates the phenol group of OTA undergoes a significant shift in pK_a upon binding to the protein (∼7.1 to 3.9), and so the high affinity site is occupied by the OTA dianion (OTA^{2-}) at physiological conditions.[26] Methylation of this site, for example, MOA, prevents such dianion formation. OTB and OHQ still possess this ionizable proton like OTA, but the charge distributions on the aromatic portion of the molecules differ, which will effect the pK_a of this group in solution as well as in the protein.

CHART 1. Structures of Monoanionic OTA and Its Derivatives



Compound	R_1	R_2
OTA	H	Cl
OTB	H	H
OHQ	H	OH
MOA	CH_3	Cl

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TABLE 1. Spectroscopic Properties of the Ligands in Aqueous Solution and in the Presence of HSA

	monoanion				dianion			HSA		
ligand	pK_a(PhOH)	A_{max}/nm, ϵ/M^{-1}·cm^{-1}	E_{max}/nm	Φ_f	A_{max}/nm, ϵ/M^{-1}·cm^{-1}	E_{max}/nm	Φ_f	pK_a(PhOH)	A_{max}/nm, ϵ/M^{-1}·cm^{-1}	Φ_f
OTA[a]	7.0−7.3	334, 6650	462	0.39	380, 10970	450	0.49	3.9	390, 11400	0.42
OTB[b]	7.8	320, 6900	461	0.17	364, 20717	400	0.42	5.3	378, 20792	0.52
OHQ[c]	8.03	350, 5952	502	0.049	396, 11808	477	0.20	6.8	350, 6200 404, 17155	0.35
MOA[d]		310, 3200	390	0.026					310, 8300	0.08

[a] pK_a values in the absence of protein from refs 23, 24, 26, and 28−30. Φ_f and binding parameters from refs 26 and 40. [b] pK_a value in the absence of protein from refs 31 and 32, and Φ_f from ref 40. [c] pK_a value in the absence of protein from ref 29. [d] Φ_f in the absence of protein from ref 40.

Experimental Section

Materials. OTB was purchased from Sigma and was used as received. MOA and OHQ were synthesized as described previously.[2,29,33−35] The dissolution rate for OTA derivatives was enhanced by dissolving the derivative in methanol (Fisher), evaporating the solvent, and adding a buffer solution of the desired pH value. Solutions were adjusted with HCl or NaOH to reach desired pH values. Extinction coefficients for derivatives in aqueous solutions were determined and are given in Table 1. Carbonate, borate, phosphate, and acetate buffers were prepared from corresponding acidic and basic components (all from Sigma-Aldrich) dissolved in HPLC water (Fisher). Buffer solutions (10 mM) with ionic strength adjusted to 0.1 by adding NaCl (Sigma-Aldrich) were used within the pH experiments while 50 mM phosphate buffer solutions at pH 7.2 were used for binding and displacement experiments. Fatty acid free HSA, sulfuric acid and 2,3,5-triiodobenzoate (TIB, Sigma-Aldrich), glycerol (VWR), and quinine sulfate (Fluka) were used as received. Buffer solutions were purged with nitrogen prior to the addition of OHQ to prevent oxidation of the derivative.[27] Recombinant domains of HSA were prepared and purified as described previously.[36] Additional Glu-Phe residues were attached at each respective N-terminus domain due to the restriction site utilized in cloning. Solutions with protein were incubated for 30 min to reach equilibrium before data were collected. All solutions except those containing HSA were purged with nitrogen prior to measuring quantum yields. The remaining experiments were performed in the presence of air at +25 °C unless otherwise stated.

Instrumentation. A diode-array spectrometer (Hewlett-Packard 8452A) was used to obtain absorption spectra. Fluorescence emission, excitation, and polarization spectra were measured with a Fluorolog 3 fluorimeter (Jobin-Yvon-Horiba). Slit widths were kept constant within each data set. All emission spectra were corrected with a correction file generated according to a previously described procedure.[37]

Data Analysis. Fluorescence quantum yield measurements used quinine sulfate in 0.1 N H_2SO_4 (ϕ = 0.546[38,39]) as a standard. Fluorescence polarization spectroscopy was used to quantify the binding affinities of derivatives with HSA, the interaction of OTA derivatives with HSA and its recombinant domains, and the displacement of derivative−HSA complexes with TIB. The relationship between the polarization value (P) of a fluorescent ligand and its binding constant to HSA has been described in eqs 2 and 3 of ref 26.

Results and Discussion

The following section is organized as follows. First, the optical properties of the molecules examined are discussed. The absorption and emission properties of these molecules are then used to quantify their binding constants to HSA and recombinant domains of HSA. These results indicated the major binding site for all molecules studied is in domain 2. To confirm the molecules occupy the same binding site, displacement studies using TIB are presented. The effects of protein on the pK_a of the ligand in the binding site are then examined. Last, a model for the binding site is proposed and the origin of the observed changes in ligand pK_a that accompany binding is discussed.

Optical Spectroscopy. The absorbance maximum of each species and the corresponding extinction coefficients are given in Table 1. The absorption maximum of the dianion is red-shifted from that of the monoanion. The emission maxima of each derivative in the protonated and deprotonated forms along with fluorescence quantum yields are indicated in Table 1. The emission maxima of OTA^{2-}, OTB^{2-}, and OHQ^{2-} show a blue shift from their respective monoanion emission maxima.

MOA lacks a proton on the phenol moiety and therefore exists as a monoanion at physiological pH values. The absorbance and emission maxima of MOA^- are blue-shifted from those of the monoanions of OTA and the other derivatives studied. Furthermore, MOA^- exhibits weaker fluorescence (Φ_F = 0.026 as compared to 0.39 for OTA^-). The large Stokes shift of the OTA^- emission is attributed to excited-state intramolecular proton transfer.[40] This reaction cannot occur upon excitation of MOA^-, and as expected, no such Stokes shift is observed. In addition, the fluorescence maxima of OTB^{2-} and MOA^- are slightly red-shifted in the presence of HSA, in contrast to the blue-shifted fluorescence maximum of OHQ^-.

When completely bound to HSA, the absorbance maxima of OTA and OTB shift to the red in comparison to that of the dianion in bulk water. Therefore, under the conditions studied, only dianion binding is observed for both OTA and OTB. The protolytic equilibrium of OHQ in aqueous solution at pH 7.2 favors the monoanionic form; the absorbance spectrum indicates an OHQ^-:OHQ^{2-} ratio of 7:1 in such a solution. The OHQ^-:OHQ^{2-} ratio in the HSA complex is 2:1. Thus, similar to the cases of OTA and OTB, binding stabilizes the dianion relative to the monoanion, but to a much lesser extent in the case of OHQ.

Binding Constants. Our past experiments[26,27] demonstrated spectral parameters of OTA changed substantially upon binding to HSA. Previously, we exploited changes present in the fluorescence polarization spectra to quantify the binding interaction between derivatives of the toxin and HSA. Polarization (P) values depend on both the fraction of bound derivative and the toxin mobility when complexed with the protein. Similar to the case of OTA, the excitation of the S_1 band of OTB^{2-} in aqueous solution results in a fluorescence spectrum exhibiting wavelength-independent polarization (P = 0.06). The microenvironment of HSA strongly retards the rotational mobility of OTB^{2-}, which is revealed by a corresponding wavelength-independent polar-

TABLE 2. Ligand Binding Constants, K_L, to Native HSA and D1−D3 in 50 mM Phosphate Buffer pH 7.2

	$K_L/10^4$ M^{-1}			
	OTA^{2-}	OTB^{2-}	MOA^{-}	$OHQ^{2-/-}$
HSA	520[a]	180	3	22
D1	1.7[a]	0.20	0.07	0.8
D2	79[a]	11	0.09	2
D3	11[a]	0.30	0.01	1

[a] Binding constants obtained from refs 26 and 47.

ization of 0.42. This polarization value is only slightly less than the limiting polarization value ($P = 0.45$) of the OTB^{2-} emission in viscous solutions of glycerol−water mixtures (9:1 v/v) at 0 °C. The rotational correlation time of HSA (ranging from 22 to 41 ns[26,41−44]) is much longer than the OTB^{2-} fluorescence lifetime (3.8 ns),[40] and the overall tumbling of the protein does not contribute substantially to depolarization. Given conditions where both protein-bound and free OTB are present, the polarization P is a measure of the fraction of bound derivative (see eq 2 of ref 26), from which the binding constant can be determined.

Similarly, polarization may be utilized to monitor the binding of MOA and OHQ. Binding constants, K_L, are summarized in Table 2 and decrease in the order $OTA^{2-} > OTB^{2-} > OHQ^{2-} \sim OHQ^{-} > MOA^{-}$.

Binding to Domains of HSA. Before one can speculate on what insights the order of binding constants provides about molecular forces responsible for stabilizing the binding of OTA^{2-} to HSA, it is important to establish that these data reflect binding to a common site in the protein and are not simply manifestations of the binding of this set of molecules to different regions of the protein. Two approaches were pursued to address whether these molecules bind to a common binding site: binding to domains of HSA and displacement studies. The domains studied were as follows: D1, residues 1−197; D2, residues 189−385; and D3, residues 381−585. These domains exhibit similar structural properties when compared to the native protein[36,41] and are valuable for determining primary and secondary binding sites, as previously demonstrated in studies examining the binding of warfarin.[36,45,46] Although the three recombinant domains are much smaller than HSA (rotational correlation times of ∼14 ns), we have previously demonstrated the binding affinities to these fragments can be determined using fluorescence polarization.[47]

All OTA derivatives displayed increased polarization values in the presence of the individual HSA domains. Table 2 indicates the respective binding constant estimates of these derivatives to D1−D3 of HSA. All molecules studied display the highest affinity for D2. A comparison of the binding affinities to D2, the fragment with the highest affinity, with those for native HSA illustrates binding constants to D2 are more than an order of magnitude smaller than those of the protein. Therefore, binding of these ligands depends to some degree on interactions between the three domains. Past work using larger fragments of HSA establishes contributions from D1 and to a lesser extent D3 are important in stabilizing the interactions between OTA^{2-} and HSA. It is reasonable to assume similar effects for the OTA derivatives studied herein. The binding affinity of OTA^{2-} to D2 is ∼7, ∼40, and ∼103 times greater than that of OTB^{2-}, $OHQ^{2-/-}$, and MOA^{-}, respectively. Thus, if these molecules share a common binding site within D2, the acidity of the phenolic group (and stabilization of the resulting dianion by the protein) is vide infra an important factor in determining the thermodynamics of ligand binding.

Displacement by TIB. To establish whether this set of molecules occupies a similar binding site within D2 of HSA, displacement by TIB was examined. TIB was chosen because X-ray crystallography has revealed the binding of TIB in site I of D2.[48] We determined the binding affinity of TIB for HSA (7.7×10^5 M^{-1});[47] however, other experiments indicate the value is slightly lower (2.0×10^5 M^{-1}).[49−51]

Previously, we demonstrated TIB displaces OTA^{2-} from its high affinity site of HSA.[47] The derivatives examined have a lower binding affinity for HSA than OTA^{2-}; thus, they will be displaced by TIB if they occupy the same binding site as OTA^{2-}. For the set of competitive displacement experiments, 1:1 ligand:HSA mixtures were prepared in the presence of [TIB]/[ligand] = 2.5−10. Steady-state fluorescence polarization of MOA^{-}, OHQ^{-}, and OTB^{2-} was then recorded. Polarization values for each derivative decreased with increasing TIB concentration, indicating displacement of the derivative from HSA by TIB. Displacement of the ligands in HSA with TIB, in addition to the binding of the ligands to individual HSA domains, confirms the derivatives share the site I binding location of OTA on HSA.

Binding Effects on pK_a. The reported pK_a values of the carboxylic group of OTA and its derivatives range from 4.2 to 4.4.[23,24,28−30] Like OTA, OTB and OHQ contain a phenolic group with pK_a values ranging from 7.0 to 8.03.[29,31,32] OHQ has a second hydroxyl group with a pK_a near 11. In the presence of oxygen and above an aqueous solution pH of 11, OHQ rapidly decomposes into a quinone.[29] Under conditions at physiological pH, both the monoanion with the protonated hydroxyl group and the dianion with the deprotonated hydroxyl group are present in aqueous solutions of OTB and OHQ. However, the monoanion species is more highly favored in the case of OHQ.

We previously noted the OTA protolytic equilibrium shifts substantially to the deprotonated form as the pK_a of the phenolic proton shifts from 7.2 to 3.9.[26] Similar absorbance shifts to the deprotonated form were observed during the binding of OTB to HSA, and to a lesser extent during the binding of OHQ to HSA. To determine an apparent pK_a value of the OTA derivatives within the protein environment, we measured absorption and fluorescence spectra for 2:1 OTB−HSA and 1:1 OHQ−HSA mixtures as a function of pH. At these concentration ratios, each derivative is present both in the aqueous phase and in the HSA environment; that is, there is noncomplete binding. The intensities of the fluorescence excitation spectra at the absorption maxima of the protonated and deprotonated OTB and OHQ were determined by collecting fluorescence at 470 and 530 nm, respectively, from solutions of different pH values. The pH profile for OTB^{2-} dianion shows two main inflection points around pH 7 and 5 corresponding to the protonation of free and bound derivatives and at approximately pH 8 and 7, respectively, for the OHQ^{2-}. Transitions around pH 4 were also apparent for the monoanion forms of the derivatives. Absorption spectra, which were also used to measure protonated and deprotonated maxima intensities for each derivative, show similar profiles. The average of the pK_a values of the derivatives in the presence of HSA from fluorescence excitation and absorbance spectra are given in Table 1. pK_a values for the phenolic proton in aqueous solution are also given for comparison.

None of the derivatives display as large a shift in the pK_a of the phenolic group as OTA. The pK_a of OTB shifts about 2.5 units while that of OHQ shifts approximately 1.2 units. As mentioned beforehand, the protolytic equilibrium of OHQ is not completely shifted to the deprotonated form, as a 2:1 OHQ^{-}:

OHQ^{2-} ratio is found upon binding to HSA. The magnitude of the pK_a shift likely reflects the stability of the dianion in the protein environment. The binding affinity, K_L, correlates linearly with the K_a of the phenolic group of the ligand bound to the protein. In all cases the dianion is more tightly bound than the monoanions, including OHQ (at pH 7.2, the ratio of monoanion to dianion shifts from 7:1 to 2:1 upon binding). MOA, which lacks the phenolic proton, cannot form a dianion and consequently has the lowest binding affinity.

The Binding Site. While the exact molecular details of the association of OTA with HSA remain to be determined, the study of derivatives of OTA provides important information. Comparison of OTA, OTB, OHQ, and MOA indicates the ionization of the phenolic group upon binding to HSA contributed significantly to the binding constant. Some insight into the molecular interactions responsible for this stabilization can be gleaned from the X-ray crystallographic structures of TIB and warfarin bound to HSA.

For the TIB−HSA complex, R257, S287, K199, and H242 are within 5 Å of the ligand.[52] Residues K199 and H242 interact with the COO^- on TIB. Comparison with the warfarin-bound structure shows the heterocylic coumarin moiety of warfarin occupies the same region as the benzyl ring of TIB. Both ligands hydrogen bond to H242 and share the site surrounded by A291, I260, I264, I290, L238, and V241.[48,53] Two important observations must be accounted for in proposing a model for the binding of OTA and its analogues. First, TIB displaces bound OTA; second, warfarin and OTA bind to site I.[27,47] The carboxylic acid moiety of TIB interacts with K199 and H242 while the O^- substituent of warfarin only interacts with H242.

Based on calculation of the OTA binding site and the displacement of OTA by TIB and warfarin, it is reasonable to postulate that the carboxylic group of OTA interacts with K199.[54] Given this placement, the isocoumarin ring of OTA would occupy the location near the benzyl moiety of warfarin. If we use the lowest energy conformation of OTA, the phenyl ring would be located in a site lined by F211, W214, and L219. In this position, residues R218 and R222 could be involved in the deprotonation of the monoanion. However, on the other side of the cavity, R257 is also well positioned to play a similar role if OTA is rotated ∼180°. We can determine the binding geometry from docking calculations. In addition, the role of R218, R222, or R257 in the deprotonation of the phenolic group of OTA can be tested by examining the binding of OTA to their respective D2 mutants. Such calculations and experiments are currently in progress.

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