Crystal structure of equine serum albumin in complex with cetirizine reveals a novel drug-binding site.

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Abstract

Serum albumin (SA) is the main transporter of drugs in mammalian blood plasma. Here, we report the first crystal structure of equine serum albumin (ESA) in complex with antihistamine drug cetirizine at a resolution of 2.1 Å. Cetirizine is bound in two sites – a novel drug binding site (CBS1) and the fatty acid binding site 6 (CBS2). Both sites differ from those that have been

proposed in multiple reports based on equilibrium dialysis and fluorescence studies for mammalian albumins as cetirizine binding sites. We show that the residues forming the binding pockets in ESA are highly conserved in human serum albumin (HSA), and suggest that binding of cetirizine to HSA will be similar. In support of that hypothesis, we show that the dissociation constants for cetirizine binding to CBS2 in ESA and HSA are identical using tryptophan fluorescence quenching. Presence of lysine and arginine residues that have been previously reported to undergo nonenzymatic glycosylation in CBS1 and CBS2 suggests that cetirizine transport in patients with diabetes could be altered. A review of all available SA structures from the PDB shows that in addition to the novel drug binding site we present here (CBS1), there are two pockets on SA capable of binding drugs that do not overlap with fatty acid binding sites and have not been discussed in published reviews.

Keywords

serum albumin; cetirizine; Zyrtec; drugs; binding pockets; crystal structure

Abbreviations

CBS – cetirizine binding site

ESA – equine serum albumin

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HSA – human serum albumin

IFE – internal filter effect

LLDF - local ligand density fit

NEG – nonenzymatic glycosylation

PBS – phosphate-buffered saline

PDB – Protein Data Bank

PEG – polyethylene glycol

pIFE – primary internal filter effect

RMSD – root-mean-square deviation

RSR – real space R-factor

SA – serum albumin

TFQ - tryptophan fluorescence quenching

TLS - translation/libration/screw

sIFE – secondary internal filter effect

1. Introduction

Serum albumin (SA) is the most abundant protein in mammalian blood plasma. Due to its high concentration and presence of multiple binding pockets, SA is a major transporter of endogenous compounds, including fatty acids, hormones, and metal ions (Peters, 1995). Many commonly used drugs such as warfarin, diazepam, and ibuprofen also bind to SA, which can be beneficial for drug delivery. Binding to SA can increase solubility, decrease the formation of aggregates, and increase the half-life of a drug. However, drugs with high affinity for SA may require higher doses to achieve the desired effect (Ghuman et al., 2005).

Previous studies of SA revealed several distinct drug binding sites (Ghuman et al., 2005). Two canonical drug binding sites were proposed by Sudlow and coworkers before a crystal structure of SA was available (Sudlow et al., 1976, 1975). As was later confirmed by multiple crystallographic studies, drug site 1 (Sudlow site I) is located in subdomain IIA, and drug site 2

(Sudlow site II) in subdomain IIIA. Recently, Wang and co-workers (Wang et al., 2013) showed that several oncological drugs bind to a site in subdomain IB, and this site was proposed to be the third drug binding site (Wang et al., 2013). In addition to the main drug binding sites, some drugs were found to bind in fatty acid binding sites (Bern et al., 2015; Ghuman et al., 2005). Crystallographic studies have revealed nine fatty acid sites on SA: seven that bind long-chain fatty acids (FA1-7) and two for short chain fatty acids (FA8 and FA9) (Bhattacharya et al., 2000b; Curry, 2009). All three main drug binding sites overlap with fatty acid binding sites: drug site 1 overlaps with FA7, drug site 2 with FA3/FA4, and drug site 3 with FA1. Therefore, all FA binding sites are considered as potential drug binding sites.

Cetirizine (IUPAC name: 2-[2-[4-[(4-chlorophenyl)-phenylmethyl]piperazin-1-yl]ethoxy]acetic acid), which is sold under the trade names Zirtec, Zyrtec, and Reactine, is a potent histamine H₁ receptor antagonist that is used to treat seasonal allergic rhinitis, perennial allergies, and idiopathic urticaria (Tillement et al., 2003). Cetirizine was the first marketed drug from the series of second-generation antihistamines showing both minimal side effects on the central nervous system and a reduced level of cardiotoxicity. Cetirizine exists physiologically as a zwitterion, and differs significantly in molecular structure compared to the first-generation H₁ receptor antagonists (Pagliara et al., 1998). It is a racemic mixture of two enantiomers, levocetirizine (R-cetirizine) and dextrocetirizine (S-cetirizine), the former being more potent (Tillement et al., 2003). At therapeutic concentrations in the blood, approximately 90% of cetirizine binds to plasma proteins, primarily to SA (Pagliara et al., 1998). There were multiple attempts to predict cetirizine binding sites on SA, though the sites predicted were inconsistent between the studies (Bree et al., 2002; Hegde et al., 2011; Liu et al., 2009; Pagliara et al., 1998). Specifically, the reports variously predicted that cetirizine bound to either drug site 1, drug site 2, or both.

In this study we present the first crystal structure of a mammalian SA, equine serum albumin (ESA, common horse), in complex with cetirizine. In this structure, cetirizine is bound in two sites that we label as CBS1 and CBS2. We determine the constants for the binding of cetirizine to CBS2 in both ESA and human serum albumin (HSA) using fluorescence tryptophan quenching (TFQ). We also compare cetirizine binding by ESA and HSA by examining the structural conservation of the binding sites. We hypothesize how cetirizine binding is affected by the presence of fatty acids and discuss the potential consequences of SA glycation on cetirizine transport. In addition, we present an overview of SA drug binding capacity based on all SA crystal structures available in PDB.

2. Material and methods

2.1. Materials

Cetirizine dihydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, LOT# 042M4707V, Catalog# C3618). ESA was purchased from Equitech-Bio (Kerrville, TX, LOT# ESA62-985, Catalog# ESA62) and HSA from Sigma-Aldrich (LOT# SLBD7204, Catalog# A3782), both as defatted lyophilized powder. After dissolving the albumin proteins in aqueous solutions, the concentrations were calculated spectrophotometrically by measuring the absorbance at 280 nm (assuming $\epsilon_{280\text{-HSA}} = 28730 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{280\text{-ESA}} = 27400 \text{ M}^{-1}\text{cm}^{-1}$, $\text{MW}_{\text{HSA}} = 66470 \text{ Da}$, $\text{MW}_{\text{ESA}} = 65700 \text{ Da}$, and path length = 1 cm) measured with a Shimadzu UV-2450 UV spectrophotometer (Kyoto, Japan).

2.2. Structure determination

2.2.1. Protein purification and crystallization

ESA was dissolved in 10 mM Tris pH 7.5 and 150 mM NaCl buffer, and was further purified using a Superdex 200 column attached to an ÅKTA FPLC gel filtration system (GE Healthcare) at 21 °C. Following gel filtration, fractions containing monomeric protein with a molecular weight of 55-60 kDa were combined and concentrated to 30 mg/mL. Crystals of native ESA were obtained by hanging-drop vapor diffusion on 24-well plates (Qiagen). Plates were set up manually and observed under the microscope. Well-diffracting crystals grew after 1 day with the reservoir solution composed of: 0.1 M Tris HCl pH 7.5, 1.8 M (NH₄)₂SO₄, 0.0875 M NaBr, 2.5% w/v PEG 8K. Crystals were soaked with a 100 mM stock of cetirizine in 100 mM Tris buffer pH 7.4 with a final concentration of cetirizine in the drop approximately 10 mM. Crystals were flash-cooled using mineral oil as a cryoprotectant.

2.2.2. Data collection, structure determination, refinement, and validation

Diffraction data for ESA in complex with cetirizine were collected at a wavelength corresponding to the selenium absorption edge (12670 eV), from a single crystal at 100 K, at the LS-CAT 21-ID-D beamline at the Advanced Photon Source, Argonne National Laboratory (Argonne, IL). The data were integrated and scaled by HKL-3000 (Minor et al., 2006), (Otwinowski and Minor, 1997). The structure was solved by molecular replacement using the native structure of the ESA protein (PDB ID: 3V08) as the template model. Initial electron density maps and model were obtained with HKL-3000 (Minor et al., 2006), which integrates MOLREP (Vagin and Teplyakov, 1997) and auxiliary programs from the CCP4 package (Winn et al., 2011). Several interactive cycles of model rebuilding and refinement were carried out by HKL-3000 interacting with REFMAC (Murshudov et al., 2011) and COOT (Emsley and Cowtan, 2004). Atomic displacement parameters were modeled using individual isotropic B-

factors with translation/libration/screw (TLS) parametrization to describe anisotropic displacement of those atoms. Seven TLS groups were introduced as suggested by the TLSMD server (Painter and Merritt, 2006). Standalone version of MolProbity (Chen et al., 2010) and the PDB validation tools (Read et al., 2011) were used for structure quality assessment. Dataset parameters and structure refinement statistics are summarized in Table 1. The atomic coordinates and structure factors were deposited in the PDB with identifier 5DQF. The diffraction images are available on Integrated Resource for Reproducibility in Macromolecular Crystallography website (<http://proteindiffraction.org/>) with doi:10.18430/M3WC7F

2.3. Tryptophan quenching assay

2.3.1. Sample preparation

Both ESA and HSA were dissolved in phosphate-buffered saline (PBS) buffer (137mM NaCl, 2.7 mM KCl, 90 mM Na₂HPO₄, and 16.2 mM KH₂PO₄, pH 7.4) and subsequently purified by size exclusion chromatography using a Superdex 200 column attached to an ÅKTA FPLC gel filtration system (GE Healthcare) at 4 °C. The fractions corresponding to monomeric protein were combined and concentrated. A portion of the purified ESA was dialyzed overnight into two additional buffers: 100 mM HEPES with 150 mM NaCl at pH 7.4, and 100 mM Tris with 150 mM NaCl at pH 7.4, and used to test the influence of the buffers on cetirizine binding. Cetirizine was dissolved in the same buffer as the protein to two final concentrations of 10 and 7.5 mM respectively, and the pH of each was adjusted to 7.4 in order to be close as possible to physiological conditions. Each of the two cetirizine stocks was subsequently diluted 1:1 with the buffer sequentially 14 times, giving a series of 30 dilutions all used for the TFQ assay. Prior to

the TFQ assay, the protein and cetirizine solutions were filtered using 0.1 µm membrane filter (Millipore, Billerica, MA, Catalog# UFC30VV00), degassed, and mixed in a 1:1 ratio.

2.3.2. Model used to determine cetirizine dissociation constant by TFQ

Since the total concentration of albumin used was significantly lower than the total concentration of cetirizine (i.e. $[P] \ll [L]$), we used a simplified model of TFQ that assumes that the concentration of free ligand is approximated by the concentration of added ligand ($[L]_{free} \approx [L]$) (Van De Weert and Stella, 2011).

$$\frac{F_0 - F}{F_0 - F_c} = \frac{[L]}{K_d + [L]} \quad (1)$$

F is the measured fluorescence, F_0 is the fluorescence of the protein without ligand, F_c is the fluorescence of fully complexed protein, K_d is the binding constant and $[L]$ is the ligand concentration. When equation 1 is solved for F , where $f = \frac{F_0 - F_c}{F_0}$ is the efficiency of quenching, we get:

$$F = F_0 \left(1 - f \frac{[L]}{K_d + [L]} \right) \quad (2)$$

However, this model does not correct for the so-called “internal filter effect” (IFE) (Van De Weert and Stella, 2011). Cetirizine does not absorb significantly at the tryptophan emission wavelength of 340 nm (secondary IFE or sIFE) and any correction concerning the absorbance of the fluorescent signal was unnecessary (Suppl. Fig. 1). However, the absorbance at the excitation wavelength 280 nm (primary IFE or pIFE) is significant, especially at higher cetirizine concentrations, and a correction had to be applied to changes in fluorescence yield upon increase in ligand concentration.

We used the correction proposed by Ehrenberg *et al.* (Ehrenberg et al., 1971) for experimental setups where all points of the sample well are read by the detector at a constant angle. This condition is true for the plate-reader with front (top) face geometry which was used in the experiment. This correction compensates for fluorescence decrease due to pIFE by multiplying the measured fluorescence by a correction factor G , defined as:

$$G = \frac{1-e^{-A_p}}{1-e^{-(A_p+A_l)}} * \frac{A_p+A_l}{A_p} \quad (3)$$

A_p is protein absorbance at 280 nm and A_l is ligand absorbance at 280 nm.

2.3.3. Tryptophan quenching measurements

Tryptophan fluorescence intensity was measured at 37 °C on a Pherastar FS (BMG Labtech, Offenburg, Germany) device using two filters: 280 nm for the excitation beam, and 340 nm for the fluorescence detection. Sample solutions with a volume of 100 µL were placed on UV-transparent, half-area 96-well plates (Corning®, One Riverfront Plaza, NY, Catalog# CLS3635). The albumin concentration of 6.1 µM for both ESA and HSA was chosen based on preliminary experiments. The cetirizine concentration varied from 0.23 µM to 5 mM. The gain value (amplification used to increase the observable signal) was adjusted to 645 and 666 for ESA and HSA respectively. The focal height was set to 6.7 mm to ensure the best signal-to-noise ratio. The albumin fluorescence intensity for each cetirizine concentration was averaged based on three independent experimental repetitions (each repetition was obtained by measuring the fluorescence of the well 10 times and averaging the acquired values). The averaged values of three independent measurements of cetirizine fluorescence in control solutions (without protein) were subtracted as a background correction for each cetirizine concentration. Experimental errors were estimated as standard deviations of the obtained values. Values of absorbance for the

calculation of pIFE correction were measured from the same samples. A_p was measured from the well with zero cetirizine concentration and $A_p + A_l$ was recorded for each cetirizine concentration in the presence of protein. Afterwards correction factor G (3) for each cetirizine concentration was calculated and the values of fluorescence intensity were multiplied by the correction factor. The TFQ model was fit by nonlinear regression method to the corrected experimental data with the Origin 6 software (OriginLab Corporation).

2.4. Sequence and structure analysis

PyMOL (Schroedinger LLC) was used to superpose the structures and generate figures. Root-mean-square deviation (RMSD) values were calculated using PyMOL's **align** function with the cycles parameter set on zero (command: align mobile_structure, target_structure, cycles=0, transform=0). PyMOL's script color_h.py was used to color-code structure by hydrophobicity according to the Eisenberg hydrophobicity scale (Eisenberg et al., 1984). BioEdit (Ibis Biosciences) was used for sequence manipulation and representation. The Mafft multiple-sequence alignment server was used to align the sequences (Katoh and Standley, 2013). In the text, the numbering of residues always refers to the HSA sequence numbering for simplicity.

2.5. Analysis of ligand binding sites on SA

The albumin structures used for the analysis of binding sites were found using three approaches. First, Dali server (Holm and Rosenström, 2010) was run using the structure of HSA (PDB ID: 1E78) to find structures with a Z-value higher than 9 (below this score no molecule was annotated as SA). The resulting list of 142 chains was further reduced to 132, by excluding protein chains with folds different than that of albumin. Second, the PDB tool was used to search

for structures with sequence similarity 30% and higher to that of HSA. The search resulted in a set of 103 structures (147 chains). Third, the PDB was searched for all records with the text ‘serum albumin’ in the molecule name field. This search produced 106 structures (149 chains). The union of all three approaches resulted in a set of 151 unique chains. Only chains with bound ligands with more than 5 non-hydrogen atoms were superposed and further analyzed (93 chains).

3. Results

3.1. Overall fold and structure quality

The crystal of ESA in complex with cetirizine adopted space group $P6_1$ and diffracted to 2.15 Å resolution. The unit cell has dimensions of $a=94.2$, $b=94.2$ and $c=141.9$ Å and accommodates one ESA monomer per asymmetric unit. The overall fold is the same as in the native structure and maintains the canonical “heart” shape. The structure may be divided into three homologous domains: I (residues 1–195, from now on residue numbering will refer to HSA), II (196–383) and III (384–585), each containing two subdomains (A and B) composed of 4 and 6 alpha-helices, respectively (Fig. 1).

The refined model has good overall geometry with a very low percentage of rotamer outliers and a low MolProbity clash score (Table 1). The structure has no Ramachandran plot outliers.

According to the amino acid sequence, the mature protein consists of 582 residues (NCBI accession code: NP_001075972), though three N-terminal residues are disordered and therefore are not visible in the electron density map. Interestingly, in contrast with the previously deposited in PDB structures of *Equus caballus* SA (PDB IDs: 3V08, 4J2V, 4OT2, 4F5U, and 4F5T), a single point mutation, R561A, is observed. The long arginine side chain cannot be modeled in this position due to steric clashes with the nearby disulfide bond connecting Cys567

and Cys558 and a symmetry-related copy of the molecule. Moreover, there is no $2mF_o - DF_c$ omit map supporting placement of the side chain. According to the NCBI database, this mutation is characteristic for *Equus ferus przewalskii*, a rare subspecies of wild horse from central Asia (accession code: XP_008524663.1). However it is possible that there is an error in the *Equus caballus* SA sequence, or the observed mutation naturally occurs in that species.

3.2. Cetirizine binding sites in equine serum albumin

Defatted ESA was used for crystallization in order to avoid potential competition of cetirizine and fatty acids for the same binding site. Two cetirizine molecules per ESA monomer were found in the crystal structure (Fig. 1 and Fig. 2). The first cetirizine binding site (CBS1) is situated in a deep pocket located at the interface between subdomains IA and IB, where cetirizine is “wedged” in a crevice between helices h2 and h3 from subdomain IA, and h2 from IB. This site is hydrophobic due to the presence of seven highly hydrophobic residues: Leu24, Phe36, Val40, Val43, Leu135, Leu139, and Leu155 (Fig. 3). The two aromatic rings of the cetirizine molecule are locked in the binding pocket due to hydrophobic interactions. Additionally, the OD1 and OD2 atoms of Asp132, which are located at the opening of the binding cavity, form a strong salt bridge to the NAF nitrogen atom of the piperazine ring and further stabilize cetirizine in this position. The carboxylate group of cetirizine protrudes outside the pocket and interacts with a symmetry-related copy of the protein. The oxygen atom OAC of the carboxylate group of cetirizine forms a salt bridge with nitrogen atom NE2 from His338 with a symmetry-related copy of the protein, which locks the tail in a defined conformation.

Cetirizine binding site 2 (CBS2) is located in a pocket formed by helix h2 from subdomain IIA and h2 and h3 from subdomain IIB. Similarly to CBS1, the two aromatic rings of the cetirizine

molecule create hydrophobic interactions with five highly hydrophobic residues which coat the pocket: Val216, Phe228, Leu327, Leu331 and Leu347 (Fig. 3). The piperazine ring is stabilized by a strong salt bridge between the NAF atom and OE1 and OE2 from Glu354. Cetirizine's carboxylate group projects outside the pocket and is bound by an electrostatic interaction with Arg209.

The C α atoms superposition of native ESA (PDB ID: 3V08) on the structure of ESA in complex with cetirizine shows a RMSD value of 1.27 Å (over 580 atoms). The superposition of all atoms of all residues that have at least one atom within 6 Å of the cetirizine molecule (atoms from native ESA superposed on atoms from ESA in complex with cetirizine) gave RMSD values of 0.78 Å (78 atoms) for CBS1 and 0.48 Å (58 atoms) for CBS2. Therefore, the binding pockets have the same conformation in both structures and there are no significant conformational changes upon cetirizine binding in either CBS1 or CBS2.

The cetirizine compound used for soaking of ESA crystals was a racemic mixture of two enantiomers, dextrocetirizine and levocetirizine, same as the marketed drug. In both cetirizine binding pockets the position of the chloride atom is ambiguous and both cetirizine enantiomers may be modeled (i.e. the aromatic rings can be swapped). Judging by the best fit to the 2mF $_o$ -DF $_c$ omit map, chloride atom B-factor, and Real Space R-factor (RSR) value we modeled only predominant conformation in each of these pockets: dextrocetirizine in CBS1 and levocetirizine in CBS2. The cetirizine molecule in CBS1 was refined with an overall occupancy of 1 and an average B-factor of 78 Å², and in CBS2 with an occupancy of 0.8 and an average B-factor of 81 Å². The two cetirizine molecules fit well to the electron density map (Fig 2) which is supported by low RSR (0.16 and 0.20 respectively) and Local Ligand Density Fit (LLDF; 1.79 and 2.04 respectively) values.

3.3. Conservation of CBS1 and CBS2 between ESA and HSA

Mature mammalian SA proteins have very high sequence conservation across different species. HSA is 84.8% similar and 76.2% identical by sequence as compared to ESA. This high sequence conservation is reflected in the conserved three-dimensional structure, and allows the protein to maintain the same functions in different species (Chruszcz et al., 2013). Since there are no major conformational changes in ESA upon binding of cetirizine, the binding sites in ESA may be directly compared to corresponding sites in the *apo*-structure of HSA (PDB ID: 1E78), after overall C α atoms superposition of the two structures. The comparison shows that the geometries of binding sites CBS1 and CBS2 are well maintained in HSA (Fig. 3.).

CBS1 in ESA is composed of 16 residues, defined as those located within 6 Å of the cetirizine molecule with side chains that are facing the pocket (Fig. 3). 10 (62.5%) of these residues are fully conserved in HSA and five (31.3%) are similar (Fig. 4). The only significant change in CBS1 is Lys136 of HSA, which corresponds to a glycine residue in ESA. Lys136 is buried inside the pocket and may potentially interfere with cetirizine binding in HSA, especially taking into account that this residue may be glycosylated in humans (see 4. Discussion). However, when comparing the structure of HSA superposed on the structure of ESA in complex with cetirizine, Lys136 does not form clashes with cetirizine molecule. Moreover, Lys136 is located at the entrance to CBS1 and demonstrates high flexibility; therefore the side chain may easily flip away from the ligand, leaving space for cetirizine to bind. The Asp132 residue forming the salt bridge with the piperazine ring of cetirizine molecule in CBS1 corresponds to Glu in HSA, which should preserve the electrostatic interaction given the conformational flexibility of the residue.

CBS2 in ESA is composed of 13 residues, defined in the same manner as for CBS1. 12 (92.3%) of them are fully conserved in HSA (Fig. 4). The only difference in HSA is Val482, which corresponds to alanine in ESA. This substitution should not create any clashes with the cetirizine molecule and may even slightly increase the propensity of CBS2 to bind cetirizine, since valine is more hydrophobic than alanine (Fig. 3). In addition, the residue forming the salt bridge with the piperazine ring in CBS2 (Glu354) is also conserved, suggesting that the electrostatic interaction is conserved in cetirizine binding to HSA as well.

3.4. Cetirizine dissociation constant at CBS2 in ESA and HSA

Both ESA and HSA have only one tryptophan residue in the entire amino acid sequence. It is located at position Trp214, which is about 33 Å from CBS1 and about 7.8 Å from CBS2. Since TFQ occurs only if the distance between the quencher and tryptophan residue is short (~5-15 Å) (Mansoor et al., 2012), TFQ is only suitable for monitoring binding of cetirizine to CBS2, because the tryptophan residue is too far away from CBS1 for its fluorescence to be disrupted by cetirizine binding in CBS1.

After applying correction for pIFE, the obtained fluorescence data fits well into the model described by the equation 1 (Fig. 5). However, the observed decrease in the intensity of fluorescence may be the result of collisional quenching. In the case of collisional quenching, the bimolecular quenching rate constant k_q cannot be larger than $2 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ (Van De Weert and Stella, 2011). The k_q calculated from obtained K_d values rules out this possibility:

$$k_q = \frac{1}{K_d \tau} = \frac{1}{300 \mu\text{M} \cdot 10 \text{ ns}} \approx 3 \cdot 10^{13} \frac{1}{\text{Ms}}$$

τ is the fluorescence lifetime of the fluorophore without ligand; in the case of tryptophan this value is in the range of 1-10 ns. Accounting for pIFE and ruling out collisional quenching leaves direct binding of the ligand as the most likely cause of decrease in fluorescence intensity. PBS buffer at pH 7.4 was selected for TFQ experiments for both albumins, since it best mimics the native blood conditions. The calculated dissociation constants K_d for cetirizine in CBS2 are $320 \pm 36 \mu\text{M}$ for ESA and $414 \pm 55 \mu\text{M}$ for HSA. We also analyzed the influence of three commonly used buffers—Tris, HEPES, and PBS, all at pH 7.4—on cetirizine binding to ESA. As shown on Fig 5, the buffers do not have a significant influence on cetirizine binding to albumin as measured by TFQ.

3.5. Comparison of CBS1 and CBS2 with other drug binding sites on SA

Contrary to the previous non-crystallographic studies, none of the cetirizine molecules are located in the main drug site 1 or in drug site 2 (Hegde et al., 2011; Pagliara et al., 1998). CBS1 does not overlap with any of the three main drug binding sites, nor the fatty acid binding sites, suggesting that CBS1 is a new drug binding site, labeled as site 4 on Fig. 6. A superposition of all available in PDB SA structures with small molecules bound showed only one structure with a drug bound in CBS1 (PDB ID: 1E7C). In the publication describing 1E7C, the interaction of halothane (general anesthetic drug) in this position was concluded by the authors to be a crystallographic artifact, as it occurs only in the presence of fatty acids and the binding was forced by the close interaction of an asymmetric copy of the protein in the crystal structure (Bhattacharya et al., 2000a). Four other small molecule ligands were found to be bound in CBS1: acetate ion, malonate ion, polypropylene glycol, and polyethylene glycol.

CBS2 overlaps with previously described fatty acid binding site FA6 (Fig. 6). Other drugs that were found in CBS2 are: halothane, ibuprofen, and 3,5-diiodosalicylic acid (a medication for the treatment of parasitic intestinal worms). Moreover, the following crystallization agents were found in this pocket: polyethylene glycol, formic acid, and malonate ion.

The superposition of all available in PDB structures of SA showed two other binding sites not corresponding to either CBS1 or the previously described main drug or fatty acid binding sites, labeled as site 5 and 6 on Fig. 6. Site 5 is characterized by a structure with the anti-cancer drug etoposide bound (PDB ID: 4LB9) and is located between subdomains IB and IIA. There is no other ligand present in this position in any other SA structure, likely due to the fact that the pocket spans on the interface of three flexible subdomains: IA, IIA, and IB. Site 6 is located on the interface between subdomains IIIA and IIIB. There are three drugs bound in this site in various SA structures: the anti-inflammatory drug oxyphenbutazone, the anti-inflammatory drug naproxen, and 3,5-diiodosalicylic acid. Other small molecules found in this pocket include the radio-contrast agent iophenoxic acid, and three molecules originating from crystallization screens: polyethylene glycol, triethylene glycol, and L-malic acid.

4. Discussion

Herein we investigated the interaction between the antihistamine drug cetirizine and ESA using X-ray crystallography and TFQ techniques. The crystal structure revealed two cetirizine binding sites on ESA: one site that constitutes a novel drug binding pocket (CBS1 or drug site 4), and the second site coincides with the known fatty acid binding site 6 (CBS2 or FA6). The binding is ensured by highly hydrophobic pockets, with hydrophilic residues additionally stabilizing cetirizine at the entrance to the binding cavities via formation of salt bridges. Analysis of the

$2mF_o-DF_c$ omit map in the vicinity of the cetirizine binding sites suggests that both cetirizine enantiomers may bind in both CBS1 and CBS2, but only the predominant molecule, dextrocetirizine in CBS1 and levocetirizine in CBS2, was modeled in the final structure.

The lack of a crystal structure of HSA in complex with cetirizine hindered direct comparison of cetirizine interaction with ESA in relation to HSA. However, structural comparison of ESA and HSA shows that the hydrophobic environment and salt bridges for both CBS1 and CBS2 are preserved in HSA due to high sequence conservation, with CBS2 in particular being virtually identical to the site in ESA. Consequently, we can expect the same mode of cetirizine binding to HSA as observed in ESA.

This conclusion is further strengthened by the results of TFQ experiment measuring cetirizine binding in CBS2 for ESA and HSA. The dissociation constants are identical within experimental error and are in the agreement with previous reports measuring the binding of cetirizine to HSA. Pagliara *et al.* observed using equilibrium dialysis two cetirizine binding sites on HSA: K_d -weak = 200 μ M, and K_d -strong = 72 μ M (Pagliara et al., 1998). Liu *et al.* observed a single cetirizine binding site on HSA with K_d = 776 μ M at 318 K by TFQ (Liu et al., 2009). Using the same method, Hegde *et al.* demonstrated that cetirizine binds to a single site on HSA and BSA with K_d = 312 μ M and K_d = 373 μ M at 308 K, respectively (Hegde et al., 2011).

Despite the significant structural conservation of CBS1 and CBS2, and similarity in the CBS2 binding constants detected by TFQ, the binding sites in the ESA crystal structure differ from those that have been previously proposed for HSA. Equilibrium dialysis of cetirizine binding to HSA in the presence of site markers warfarin (drug site 1) and diazepam (drug site 2) indicated that both sites are involved in cetirizine binding (Pagliara et al., 1998). Pagliara *et al.* also concluded that cetirizine binds with higher affinity to drug site 2 and lower affinity to drug site 1

(Pagliara et al., 1998). Bree *et al.* showed that levocetirizine binds to three different binding sites on HSA: one strong site overlapping with drug site 2 and two weak sites with very low, non-specific affinity. In the same study, the binding of levocetirizine to HSA was also shown to decrease in the presence of fatty acids, which was concluded to be consistent with levocetirizine binding to drug site 2 (Bree et al., 2002). An independent study of cetirizine and levocetirizine binding to HSA using TFQ detected just one binding site in which levocetirizine was shown to compete with diazepam (drug site 2) for binding (Liu et al., 2009). Conversely, a recent study of cetirizine binding to BSA and HSA using TFQ found that cetirizine bound primarily at drug site 1 (Hegde et al., 2011).

These discrepancies have two feasible explanations: a) the actual sites for cetirizine binding on SA revealed by the crystal structure of ESA could not be resolved using other methods, or b) HSA has different binding sites for cetirizine than ESA despite the level of sequence and structure conservation and similarity of binding constants. Differences in binding sites for HSA versus SA from other mammals have been reported for other ligands (Sekula et al., 2013). This raises the issue of potential differences in drug transport and delivery between human and animal models used in clinical studies, and highlights the importance of structural studies of albumins from species other than human.

Nonenzymatic glycosylation (NEG or glycation) results in the addition of reducing sugars and/or their reactive degradation products to proteins in blood plasma (Anguizola et al., 2013). This process is promoted by the presence of elevated blood glucose concentrations in patients with diabetes, and affects various proteins including HSA (Anguizola et al., 2013). In certain diabetic states, the binding of drugs to HSA can be significantly altered (Ruiz-Cabello and Erill, 1984). In addition, levocetirizine was indicated to bind more strongly to glycated HSA than to non-

glycated HSA (Liu et al., 2009). An analysis of lysine and arginine residues in the context of glycation indicates that several residues in CBS1 and CBS2 may potentially be glycated. There are two such residues in ESA facing the ligand in the CBS1 pocket, Lys17 and Lys20. Moreover, HSA has one additional lysine sticking into the pocket, Lys136. Among these residues, Lys20 and Lys136 have been shown to be glycated in HSA *in vitro* (Gadgil et al., 2007). In the CBS2 pocket, there are two residues, Arg209 and Lys351, that could be potentially glycated, and both were reported to undergo glycation in HSA (Anguizola et al., 2013; Barnaby et al., 2011). We conclude that for patients with diabetes the affinity of cetirizine to SA can be affected by SA glycation. Although the exact effect of SA glycation on cetirizine binding is still unknown, it may be necessary to adjust the amount of administrated drug for patients with diabetes in order to achieve the same therapeutic effects.

Our review of all crystal structures of SA in complex with small molecules available in the PDB shows that in addition to the novel drug binding site CBS1, there are two other drug binding sites (Fig. 6, drug sites 5-6) that do not overlap with fatty acid binding sites. Consequently, the drugs bound in these sites will not compete directly with fatty acids for transport on albumin. On the other hand, the main drug binding sites 1-3 on SA do bind fatty acids, and therefore drug binding in these sites (as well as in all other FA binding sites including CBS2) can be affected by competition with fatty acids. Elevated levels of fatty acids (from dietary intake, diabetes mellitus, or myocardial infarction) or decreased levels of albumin (liver disease) (Spector et al., 1969) may decrease the apparent binding of drugs in these sites.

The results presented here describe the interaction between SA and the antihistamine drug cetirizine on the molecular level, contribute to further understanding of drug transport and delivery in mammals, and potentially will inform further rational drug design.

Glossary

- R value - is a measure of the agreement between the crystallographic model and the experimental X-ray diffraction data
- R_{free} value – is a measure of how well the current atomic model predicts a subset of the measured reflection intensities that were not included in the refinement
- B-factor – is a measure of how much an atom oscillates or vibrates around the position specified in the model
- RMSD - is the measure of the average distance between the atoms of superposed proteins
- Space group – is a designation of the symmetry of the unit cell of a crystal
- Unit cell - is the most basic and least volume consuming repeating structure of a crystal
- TFQ - refers to any process which decreases the fluorescence intensity of a tryptophan

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Figure 1. (a) The overall fold of equine serum albumin structure in complex with cetirizine.

Helices are represented by ribbons, and cetirizine molecules are shown in green in stick

representation and marked with red squares. (b) Topology diagram of albumin. Helices are represented by cylinders, and loops by arrows. Subdomains are colored according to the legend.

Names of helices take the form ‘h1’, where ‘h’ stands for helix and numbers indicate the order of the helices in each subdomain.

Figure 2. Cetirizine binding sites in ESA. (a, b) CBS1, (c, d) CBS2. Cetirizine molecules are

shown in sticks, carbon marked in green, chloride in orange, nitrogen in blue, and oxygen in red.

Helices are marked by the subdomain name and helix number as proposed in (Sugio et al., 1999).

(a, c) Cetirizine shown in its binding environment. The $2mF_o - DF_c$ omit electron density maps are

presented in orange ($\sigma = 0.8$). (b, d) The protein surface in the cetirizine binding environment is

color-coded by hydrophobicity. Color scales from red to white, where red indicates more hydrophobic and white more hydrophilic.

Figure 3. Superposition of cetirizine binding residues from ESA (white) and HSA (blue, PDB

ID: 1E78) in CBS1 (a) and CBS2 (b). Cetirizine molecules are shown in sticks, carbon marked in green, chloride in orange, nitrogen in blue, and oxygen in red.. Residues that are not conserved

between ESA and HSA are labeled with the ESA residue name followed by the HSA residue number and the HSA residue name. The salt bridges between cetirizine and ESA are depicted with dashed yellow lines.

Figure 4. Sequence alignment of HSA and ESA. Residues forming the CBS1 in ESA are marked in green, and CBS2 in red. Residues that are conserved in HSA are highlighted in a darker shade and similar residues are in a lighter shade. Among them, highly hydrophobic residues are marked with rectangles. The arrows indicate residues involved in cetirizine binding and shown to undergo glycation in certain conditions (such as diabetes).

Figure 5. TFQ in ESA and HSA by cetirizine. Graphs show tryptophan fluorescence intensity after applying correction for pIFE vs. cetirizine concentration. Error bars represent standard deviation of fluorescence intensities from three independent replicates of the experiment. TFQ in ESA (a) and HSA (b) as a function of cetirizine concentration in PBS buffer. (c) Influence of three different buffers on cetirizine binding in ESA: HEPES, Tris, and PBS. F_0 – fluorescence of protein without ligand, K_d – binding constant in μM , and f – the efficiency of quenching.

Figure 6. Summary of the ligand binding capacity of SA based on all crystallographic structures of SA currently deposited in PDB. Helices are shown in ribbon representation, and ligand atoms in spheres. The name of each binding site is labelled, CBS in red, drug binding sites that do not overlap with known FA binding in blue. All of the atoms of all ligands found in a particular site are colored in one color.

Table 1. Crystallization data and refinement statistic of ESA in complex with cetirizine.

⁺Values in parentheses are for the highest resolution shell.

[#] $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean of I observations $I_i(hkl)$ of reflection hkl .

[‡] R_{work} and $R_{free} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively, calculated for working set (R_{work}) and for 5% of the data, which were omitted from refinement (R_{free}).

Figure 1

Figure 2

Figure 3

(a) CBS1

(b) CBS2

Figure 4

Figure 5

Figure 6

Table 1

ESA in complex with cetirizine	
PDB ID: <u>5DQF</u>	
Data collection	
Wavelength (Å)	0.98
Space group	P6 ₁
Unit-cell parameters (Å, °)	a = 94.2, b = 94.2, c = 141.9 $\alpha = \beta = 90, \gamma = 120$
Resolution (Å)	80.00 - 2.15 (2.19 - 2.15) ⁺
Completeness (%)	99.8 (100.0)
Total number of reflections	297500
No. of reflections (refinement/R _{free})	36754/1879
Mean I/σ(I)	35.6 (2.3)
CC ½ - highest resolution shell	(0.79)
Redundancy	7.7 (7.7)
R _{merge} [#] (%)	0.07 (0.95)
Structure refinement	
R _{work} [‡] (%)	18.9
R _{free} (%)	24.1
Bond lengths RMSD (Å)	0.01
Bond angles RMSD (°)	1.4
Mean B value (Å ²)	38
Number of protein atoms	4408
Mean B value for protein atoms (Å ²)	55

Number of water molecules	248
Mean B value for water molecules ($\text{\AA}^2$)	57
Number of ligand/ion atoms	124
Mean B value for ligand/ion atoms ($\text{\AA}^2$)	70
Clashscore	1.45
Clashscore percentile (%)	100
Rotamer outliers (%)	0.42
Ramachandran outliers (%)	0
Ramachandran favored (%)	98.44
Residues with bad bonds (%)	0
Residues with bad angles (%)	0
MolProbity score	0.88