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

Katarzyna B. Handing[a,b], Ivan G. Shabalin[a,b], Karol Szlachta[a,c], Karolina A. Majorek[a,b], Wladek Minor[a,b,*]

[a] Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA 22908-0736, USA
[b] New York Structural Genomics Research Consortium (NYSGRC), USA
[c] Faculty of Physics, Warsaw University of Technology, 00-662 Warszawa, Poland

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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.

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 et al. 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 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. 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.

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.

* Corresponding author.
E-mail address: wladek@iwonka.med.virginia.edu (W. Minor).

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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 H_1 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 H_1 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 $\varepsilon_{280\text{-HSA}} = 28730\ M^{-1}\ cm^{-1}$, $\varepsilon_{280\text{-ESA}} = 27400\ M^{-1}\ cm^{-1}$, $MW_{HSA} = 66470$ Da, $MW_{ESA} = 65700$ 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: 100 mM Tris HCl pH 7.5, 1800 mM $(NH_4)_2SO_4$, 87.5 mM NaBr, 2.5% w/v PEG 8 K. 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 http://dx.doi.org/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 (137 mM NaCl, 2.7 mM KCl, 90 mM Na_2HPO_4, and 16.2 mM KH_2PO_4, 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).

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