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Crystallographic studies of the complexes of bovine and equine serum albumin with 3,5-diiodosalicylic acid

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ABSTRACT

Due to their extraordinary binding properties, serum albumins are the main transporters of many small molecules in the circulatory system. Although all mammalian serum albumins exhibit quite high sequence similarity, their binding abilities are not the same. Until now, only human serum albumin (HSA) was subjected to extensive structural studies in complexes with various ligands. Here we present two crystal structures of the complexes of equine and bovine serum albumins with 3,5-diiodosalicylic acid (DIS), at resolutions 2.12 Å and 2.65 Å, respectively, and analyze interactions of the DIS ligand with both macromolecules. We highlight the differences in distribution of DIS binding sites between the bovine and equine serum albumins and compare results with the HSA binding ability of DIS and other structurally similar ligands.

1. Introduction

The heart-shaped and highly helical structure of serum albumin possesses a number of binding pockets. This three-domain molecule, with a characteristic pattern of 17 disulfide bridges, has originated by gene triplication of its one-domain precursor [1]. Each of the three homologous domains (I–III), further grouped into two subdomains (A and B), has evolved specific binding properties [2]. Although serum albumin domains exhibit extensive structural homology, the distribution of their binding sites is not the same. Two main binding regions within domains II and III, named Sudlow's sites [3,4] or drug binding sites (DS1 and DS2), are responsible for interactions with the ligands which present a dual character – lipophilic and anionic at the same time. The compounds transported by albumin include metabolites, waste products, toxins and many drugs [5]. There are seven high-affinity binding locations common for long-chain and medium length fatty acids (FA1–FA7) and several with lower affinity to FA with medium and short chains [6]. Only a single binding site is found in the HSA complexes with ligands such as heme [7], whereas up to five binding sites are identified in complexes with thyroxin [8]. Binding of different types of ligands within a serum albumin can be competitive or cooperative and often is stereospecific [9]. Ligands bound to serum albumin are transported through the circulatory system to their place of function or disposal, while remaining harmless, with no threat of precipitation or undesirable effects.

Salicylic acid and its derivatives are used as components of pharmaceutical products, dyes, flavors, and preservatives. The compound, 3,5-diiodosalicylic acid (2-hydroxy-3,5-diiodobenzoic acid, DIS), is used as an intermediate for veterinary anthelmintic agents (*closantel* and *rafoxanide*) [10,11], a medication capable of causing the evacuation of parasitic intestinal worms. DIS was also studied as an agent preventing amyloid fibril formation from transthyretin (TTR) aggregates (PDB ID: 3B56) [12]. According to the crystallographic study of that complex, DIS shows high affinity to the inner halogen pocket of TTR and therefore possesses better stabilizing properties of the TTR tetramer than other salicylates. Due to the presence of iodine atoms, DIS is also used as a source of heavy atom phases in crystallographic investigations of the new crystal structures of proteins and, at the same time, is a good ligand for analyzing protein binding abilities. Complexes of albumins with iodine derivatives of salicylic acid were studied previously for HSA [13–15] and ESA [16].

Interactions between proteins and halogen-containing small molecules have been recently described to have a great value in the binding mode of drugs in protein–drug complexes [17]. The charge distribution of the halogen atoms covalently bound to the carbon in C—X moieties (X – halogen atom) is not even. A region of positive electrostatic potential, called "σ-hole", is formed at the tip of the C—X bond and the belt-like negative charge coaxial with the C—X bond. This gives the halogen atoms a possibility to act not only as donors via the "σ-hole", resulting in formation of a halogen bond, but also as acceptors of a hydrogen bond [18]. Halogen bonds are usually co-linear to the C—X bond, with the angle to the C—X in the range of 140–180°, whereas hydrogen bonds are roughly perpendicular to the C—X bond. Due to the complexity of interactions

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in macromolecular systems and also the resolution limitations of protein crystal complexes, the geometry of the protein–halogen interactions may appear to be disturbed.

Here we characterize the binding abilities of equine and bovine serum albumins based on the crystal structures of BSA–DIS (PDB ID: 4JK4) and ESA–DIS (PDB ID: 4J2V) complexes with 3,5-diiodosalicylic acid. Despite the high level of sequence homology among BSA, ESA, and HSA (over 70% sequence identity and almost 90% sequence similarity), a comparison of these structures with an analogous complex of HSA reveals numerous differences in the mode of binding of DIS. A molecule of DIS is bound in BSA and ESA in four places, not necessarily the same in both albumins. In the case of HSA there are reports indicating two binding sites of DIS without fatty acids [13] and only one DIS site in the presence of fatty acids (PDB ID: 2BXL) [15].

2. Materials and methods

2.1. Materials

Lyophilized equine serum albumin was purchased from Equitech-Bio Inc. (Kerrville, TX). Bovine serum albumin, 3,5-diiodosalicylic acid, calcium acetate, mmePEG 5 K and buffer components were purchased in Sigma–Aldrich (St. Louis, MO, USA). Crystal screens, crystallization plates, and Tacsimate™ pH 6.0 were bought from Hampton Research (Aliso Viejo, CA, USA).

Albumins were purified according to the procedure used for the native serum albumins [19] that involved two steps: an activated charcoal treatment and gel-filtration chromatography on an AKTA FPLC system (Amersham Biosciences, Uppsala, Sweden). FPLC eluents of BSA and ESA were concentrated on Vivaspin filters (Sartorius, Göttingen, Germany) using a centrifuge (Eppendorf, Hamburg, Germany).

2.2. Methods

2.2.1. Preparation and crystallization of the complexes of bovine and equine serum albumins

The complex of ESA with 3,5-diiodosalicylic acid was formed by mixing defatted and purified albumin [19] at 1 mM (67 mg/ml) concentration in buffer (100 mM NaCl and 10 mM Tris at pH 7.4) with 10-molar excess of DIS, which was added in the form of 200 mM solution in ethanol. The mixture was incubated overnight in 28 °C with stirring and was centrifuged before crystallization setup.

Complexes of BSA with DIS were obtained by soaking native crystals grown in two crystallization conditions: (1) 17% PEG MME 5 K, 175 mM NH_4Cl, 100 mM MES buffer at pH 6.5 and (2) 0.2 M calcium acetate, 24% PEG MME 5 K and 0.1 M MES pH 6.5 with DIS added to the drop. ESA–DIS complex was cocrystallized in 85% Tacsimate at pH 6.0 and large enough crystals grew after 2–3 days in 20 °C. BSA–DIS crystals grew significantly slower, over the course of several weeks. The vapor diffusion hanging drop method was used for crystallization of both proteins.

2.2.2. Data collection, structure determination and refinement

X-ray diffraction data were collected using synchrotron radiation from a single crystal of the ESA–DIS complex (2.12 Å), at 100 K, on X-12 beam line at DESY in Hamburg, Germany. Initial data of the BSA–DIS complex (3.08 Å), obtained from crystals grown in (1) crystallization conditions, were collected on line I-911 at MaxLab in Lund, Sweden. Final data of the BSA–DIS complex (2.65 Å) from crystals grown in (2) were collected on BL14.2 at BESSY Berlin, Germany [20]. The ESA–DIS complex was crystallized under conditions which provided good cryoprotective properties [21], thus the crystal was directly flash frozen in the nitrogen cryogenic stream. For the BSA–DIS crystal a special mounting procedure, preventing the ice-ring formation during vitrification, was used; crystals were fished from the crystallization drop and the excess of the mother liquid was removed to create a thin film [19].

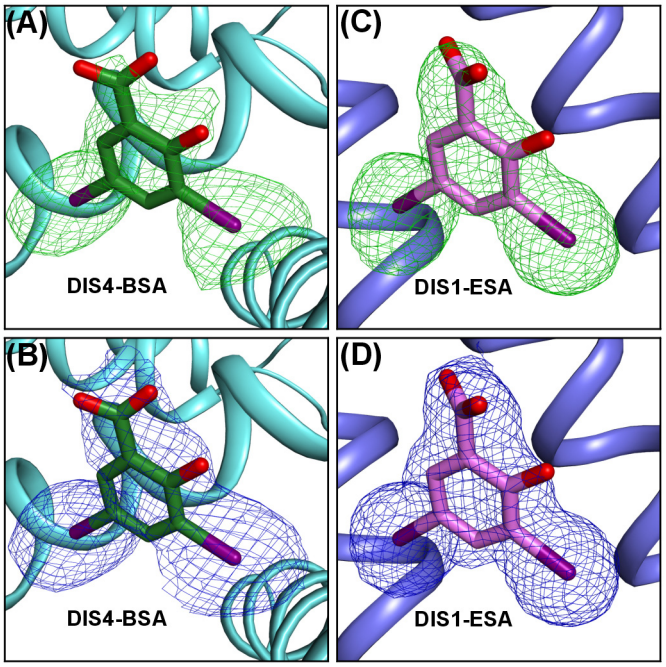

Fig. 1. Examples of omit maps $F_o - F_c$ (A and C) at 2.7σ and $2F_o - F_c$ (B and D) at 1.0σ for DIS4 in BSA (A and B) and DIS1 in ESA (C and D) complexes.

Hexagonal crystals of ESA–DIS belong to the space group $P6_1$ with a single molecule of ESA in the asymmetric unit; the BSA–DIS complex crystallized in a monoclinic system, space group $C2$, with a dimer in the asymmetric unit. The diffraction data were processed with XDS [22]. Both structures were solved by molecular replacement [23] in MOLREP [24] against *apo* structures of ESA (PDB ID: 4F5U) and BSA (PDB ID: 4F5S).

The F_o–F_c maps clearly showed positive electron density corresponding to the 3,5-diiodosalicylic acid molecules in both complexes (Fig. 1). The positive peaks for iodine atoms were very strong (from 10 up to 40σ) and left no doubt as to their positions. Additionally, to confirm the location of DIS binding sites, the anomalous signal of the iodine atoms (2.9 e at the experimental wavelength) [25] in the diffraction data was used. To generate the anomalous maps the diffraction data were reprocessed and the CAD and FFT programs were used [26]. Both structures were refined initially with Refmac5 [27,28] from the CCP4 package [29] and further refinement was continued in PHENIX [30], with manual rebuilding in Coot [31]. Program PROCHECK [32] was used to assess the quality of the final models. A summary of data collection and refinement statistics are given in Table 1.

2.3. Accession numbers

Coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers PDB ID: 4J2V for ESA–DIS and PDB ID: 4JK4 for BSA–DIS.

3. Results and discussion

3.1. Binding sites of the 3,5-diiodosalicylic acid in the complexes with BSA and ESA

Defatted ESA and BSA bind four molecules of 3,5-diiodosalicylic acid each (Fig. 2). In both complexes, two DIS molecules are bound