STRESZCZENIE ROZPRAWY DOKTORSKIEJ

„Badania strukturalne oddziaływania wybranych związków trójcyklicznych z nowymi wariantami β-laktoglobuliny posiadającymi modyfikacje w rejonie kieszeni wiążącej”

(„Structural studies of the interaction of tricyclic compounds with new β-lactoglobulin variants possessing a modifications in the binding site region”)

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Bovine β-lactoglobulin (BLG) belongs to the family of lipocalins - a group of various and small proteins that typically bind or transport chemically sensitive and poorly soluble biological compounds. Lipocalins have a simple structure; through a combination of genetic engineering and molecular selection techniques, it is possible to modify these proteins to change their selectivities and affinities for ligands. The main BLG binding site is the hydrophobic interior of the β-barrel. Introducing mutations inside the barrel makes it possible to obtain proteins with completely new properties and affinity for ligands.

The aim of this work was to design and obtain new variants of β-lactoglobulin with modified binding pockets and to study how selected mutations affect the protein's ability to interact with the tested low-molecular-weight ligands. Using site-directed mutagenesis, new BLG variants were obtained: F105L/L39A/M107W (LAW), I56F/L39A/M107W (FAW), I56F/L39A/I71W (FA71W), and L58F/V92Y (58F/92Y). They contained additional aromatic residues introduced in the region of the hydrophobic binding pocket, which affected the identification and binding of drugs containing aromatic fragments. Molecules containing the tricyclic system, for example, antipsychotics and antidepressants, which are often overdosed in suicide attempts, were chosen as model ligands. They can be divided into four groups: (1) tricyclic drugs with an aliphatic chain: amitriptyline (AMT), clomipramine (CLO), doxepin (DOX), (2) tricyclic drugs containing a cyclic aliphatic fragment: ketotifen (KET), amoxapine (AMX), (3) ligands containing a phenothiazine group: fluphenazine (FLU), chlorpromazine (CPZ), and (4) cetirizine (CET). All of the new variants were also tested for their affinity for fatty acids (palmitic or myristic), which are considered as a natural BLG ligands.

In this study, recombinant proteins obtained by production in bacterial expression systems were used. Proteins were purified by chromatographic methods using Fractogel and Sephadex resin and crystallized by hanging drop vapor diffusion technique. To optimize the crystallization process, different concentrations of protein and precipitants were tested. Diffraction measurements were made using Supernova and Synergy-S (Rigaku Oxford Diffraction) diffractometers and synchrotron.

Good quality crystals suitable for diffraction measurements were obtained for almost all protein-ligand complexes. Crystallization studies showed that the best conditions for the growth of good-quality crystals were 2.4-2.8 M ammonium sulfate in 0.5 M Tris-HCl pH 8.5. Trigonal crystals with $P3_221$ space group symmetry were obtained. The asymmetric unit contained a single protein chain.

Structural analysis of the complexes showed that the shape of the binding pocket in the apo form of the new BLG variants, containing tryptophan residues in the modified β-barrel, determines the selectivity of ligand binding. The lowest selectivity was demonstrated by FAW variant, which binding almost the entire group of ligands tested in this work (AMT, CLO, DOX, KET, FLU and CPZ). Binding of ligands inside FAW does not cause conformational changes in the amino acid sidechains, which indicates that the binding pocket has a geometry capable of accommodating a single drug molecule, regardless of its size and shape.

The most selective is the LAW variant, which bound only drugs containing the phenothiazine group: fluphenazine (FLU) and chlorpromazine (CPZ). In the apo form of this variant, it was noted that the starting position of Trp107 significantly flattens the binding site and blocks access to the hydrophobic interior of the β-barrel. The CPZ molecule conformed to the shape of the pocket, while the much larger tricyclic FLU system with an attached -CF_3 group forced a conformational change in Trp107. The FA71W variant showed selectivity intermediate between LAW and FAW. Ligand binding forced a position change, but without a conformational change of the Trp71 side chain. These changes were induced by ligands with relatively rigid tricyclic system (ligands from group 2, 3 and CET). Ligands from group 1 (AMT, CLO, DOX) were not rigid enough to be bound inside the FA71W barrel.

Among the 58F/92Y complexes, the presence of ligand was confirmed only in the structure of the complex with amoxapine (AMX). The binding pocket of the 58F/92Y variant is less flexible than in the other BLG variants. The localization of the L58F and V92Y mutations prevents conformational changes of the side chains and adaptation of the binding pocket to the shape of the ligand. The 58F/92Y mutant accepts only molecules that conform in shape and size to the binding pocket architecture.

One of the main goals of the selected mutations was to shorten the binding pocket and prevent the binding of fatty acids, which are considered BLG's natural ligands. The results showed that the selected mutations introduced in the BLG binding pocket, change not only its geometry but also its affinity for ligands. Crystallization trials showed the lack of ligand in the binding site. Fatty acids present in the cytoplasm during expression or secreted from damaged cell membranes at the purification stage cannot spontaneously bind in the binding pocket of the new BLG variants, so they do not interfere with the binding of target ligands, i.e. tricyclic drugs. The introduced mutations therefore prevent this phenomenon and increase the probability of binding the drug molecule.

The main research method used in this work was X-ray crystallography. This was complemented by circular dichroism (CD) spectroscopy, which allowed estimation of the dissociation constant (K_d) for selected protein-ligand complexes, and nanoscale differential fluorescence (nanoDSF), which allowed determination of the thermal stability (T_m) of mutants. The affinity of ligand binding by the protein was expressed by K_d value. The results showed that these values are an individual property of the protein-ligand pair, depending on the type and number of interactions and complementarity of the shape of the β-barrel and the ligand. The highest binding affinity and binding specificity were observed for FA71W-FLU and FA71W-CPZ complexes, in which the presence of relatively specific R-Cl---π and R-CF_3---π interactions and π---π (edge-to-face) interactions between the ligand molecule and protein residues was confirmed. Thermostability measurements showed that the thermal stability of the new BLG variants is strongly affected by mutations introduced in the middle part of the β-barrel; I56F and V92Y mutations strongly decrease the stability of the protein. Replacing Phe107 with leucine has a stabilizing effect on the protein and increases its denaturation temperature.

Recombinant proteins are a growing group of innovative therapeutic agents. The new BLG variants presented in this work, particularly FAW and FA71W, appear to be promising drug carriers. They possess a unique combination of properties that could provide a great basis for further studies on ligand binding selectivity. Enhancing the thermostability of these variants requires further work, which will increase the probability of their potential application in medicine.