Structural analysis of the disease-causing variants of the human dihydrolipoamide dehydrogenase and the inclusion complexes between sanguinarine and cucurbit[7]uril

Ph.D. thesis booklet

Réka Mizsei

Semmelweis University
János Szentágothai Doctoral School of Neurosciences

Supervisor: Dr. Ambrus Attila, Ph.D., Associate Professor

Official reviewers:
Dr. Béni Szabolcs, Ph.D., Associate Professor
Dr. Gáspári Zoltán, Ph.D., Associate Professor

Head of the examination Committee:
Dr. Noszál Béla, D.Sc., Full Professor

Members of the examination committee:
Dr. Bodor Andrea, PhD., Assistant Professor
Dr. Mészáros Tamás, PhD., Associate Professor

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Introduction

How molecular features such as the structure, dynamics and intermolecular interactions do relate to the macroscopic function? In this thesis I studied two main topics related to structure and function relationship: the first part discusses the inclusion complex formation between small organic molecules; the second part investigates the structural features of the pathogenic variants of a versatile enzyme protein, the human dihydrolipoamide dehydrogenase.

The sanguinarine (SA) is a natural benzo[c]phenanthridine alkaloid, which exhibits anticancer, antimicrobial, antifungal properties and interacts with nucleic acids. The main challenges in the applicability of SA are its low solubility and photostability, which is related to susceptibility of SA to nucleophilic addition of OH. We worked together with the Biczók Laboratory where they recognized that cucurbituril can increase the photostability of SA. Cucurbit[n]urils (CBn) are macrocycles composed of n glycoluril units that can form inclusion complexes with organic small molecules. Inclusion complex is a chemical complex in which one chemical compound (the "host") has a cavity into

which a "guest" compound can be accommodated. Until our research there were no structural information on the stoichiometry and structure of the SA-CB7complexes.

The human dihydrolipoamide dehydrogenase (hE3) is a pyridine nucleotide-disulfide oxidoreductase, which was primarily recognized as an important enzyme in the metabolism of sugars and amino acids being the common subunit of the mitochondrial α-keto-acid dehydrogenase complexes and the glycine cleavage complex. However, hE3 plays versatile roles in our bodies; besides its dehydrogenase function hE3 may function as a diaphorase and oxidase with various electron acceptors, and hE3 also shows proteolytic activity. Although the mechanism by which hE3 can modulate its multiple activities was not entirely understood, it is clear that single amino acid mutations can lead to serious disease in human patients. The symptoms of hE3 deficiency result in a great deal of clinical heterogeneity and clinical phenotypes do not completely correlate with the loss of dihydrolipoamide dehydrogenase activity, suggesting alternative disease mechanisms of the 14 disease-causing hE3 variants reported to date. Before our work no experimental structural information of the pathogenic hE3 variants was available; our

knowledge of the pathomechanism of the hE3 deficiency were limited to biophysical essays and the crystal structure of the wild type hE3. The hE3 is a functional 100 kDa homodimer, each monomer binds tightly, but non-covalently, a single FAD prosthetic group in its catalytic pocket.

Objectives

Characterization of the sanguinarine (SA)-cucurbit[7]uril (CB7) inclusion complexes. We aimed to describe how CB7 stabilizes SA even though the size differences do not allow CB7 to encapsulate the entire SA molecule. Reviewing the literature of CB7 complexes left us with numerous unanswered questions: Does SA get encapsulated or the cationic SA^{+} form only coordinates to the CB7? Is there an end of SA that preferentially gets encapsulated or both ends have similar affinities to CB7? Can be any higher order macromolecular organization observed in solution? My goal in the present study was to investigate the host-guest interactions in biological relevant dilute solutions. I designed NMR experiments that would reveal the size, stoichiometry and structure of the complexes present in solution. Also, we aimed to characterize the thermodynamics between the species in solution by NMR.

Structural analysis of the hE3-defficiency. The main objectives of the thesis were to produce and structural characterize the 14 pathogenic hE3 variants. First, I wanted to develop a protein expression and purification method of hE3 variants based on the

previous periplasmic expression, while switching to higher yield cytosolic expression bacterial system. I seeked to investigate the changes induced by the pathogenic mutations in the hE3 both in the protein dynamics in solution by hydrogen-deuterium exchange mass spectrometry (HDX MS) and in their crystal structures. I planned to develop a general crystallization protocol, including exploring the effect of soaking with ligands, composition of the cryoprotectant, optimizing the diffraction parameters, the data processing and acquisition.

Methods

In the first part of my thesis I used liquid phase nuclear magnetic resonance (NMR) spectroscopy not only to collect structural information on organic molecules, but to determine kinetic and thermodynamic parameters of the equilibrium system. I also described the hydrodynamic radius of the species in solution via NMR based diffusion measurements. The experimental molecular sizes agreed with the molecular sizes calculated using semiempirical molecular modeling.

To investigate the solution dynamics of proteins, we utilized hydrogen-deuterium exchange mass spectrometry (HDX MS). In the light of the previously published crystal structure of hE3 we analyzed the possible sources of solvent accessibility in certain regions of the hE3 homodimer. Also, we followed the changes induced in the HDX pattern of hE3 by the pathogenic amino acid substitutions.

The third structural method in the thesis is the protein crystallization and single crystal X-ray crystallography. Illustrated by the example of hE3 and its variants I describe

various manual and automatized crystallization techniques, and I discuss the relationship between the greater degree of thermal stability and crystallization behavior.

During my doctoral project I got practical experience in the general molecular biology methods such as generating protein variants with recombinant DNA technology using bacterial cells, protein purification with chromatographic and electrophoresis techniques and measuring protein melting curves by thermofluor measurements.

Results

1. Using NMR spectroscopy, I demonstrated that SA and CB7 readily form inclusion complexes with stoichiometries of 1:1 and 1:2
2. Based on the concentration ratios measured in the ^{1}H NMR spectra I determined the equilibrium constant of the 1:2 SA:CB7 complex formation as $K_2 = \frac{[1:2]}{[1:1][CB7]}$ = 2900 L/mol.
3. Besides collecting concentration and temperature dependent ^{1}H NMR spectra I applied ^{1}H-^{1}H NOESY measurements to define the geometry of the SA:CB7 inclusion complexes. I identified that the I end of SA is preferentially incorporated into CB7.
4. Using ^{1}H DOSY NMR measurements I determined the hydrodynamic radii of the 1:1 and 1:2 SA:CB7 inclusion complexes (7.5 ± 0.7 Å; 8.6 ± 0.8 Å). The experimental values were in concordance with the semiempirical molecular modelling results, which showed only 15% increase in the hydrodynamic radius when the 1:2 complex is formed from the 1:1.

5. I optimized the bacterial expression and affinity chromatography purification protocol for large scale production of hE3 and its pathogen variants.

6. By hydrogen-deuterium exchange mass spectrometry (HDX MS) I evaluated the solvent accessibility of the peptic peptides of the wild type hE3. I analyzed the solution structure and dynamics of hE3 while comparing our HDX MS results to the crystal structures of hE3 published previously. In agreement with the crystal structure, no significant HDX was measured for the central core peptide segments of the hE3 dimer (261-274, 434-441, 459-464); while the non-structured peptide segments on the surface of the hE3 homodimer (275-289, 339-351) exhibited the highest deuterium incorporation yields. The HDX MS data on the 81-85 peptide segment implied deviation of the solution structure from the configuration observed in crystal. I propose that the extended configuration of the 81-85 arm in the crystal structures is caused by the crystal packing, as 81-85 forms crystal-stabilizing interdimer contacts. Our data suggests the residues 81-85 should be buried from the solvent by being in a more retracted conformation in solution.

7. The structural and alterations induced by ten disease-causing hE3 variants relative to the wild type protein was

investigated by HDX MS. Each pathogenic variant of hE3 exhibited a unique HDX pattern. The mutual feature of the data sets was that none of the variants showed increased HDX in the dimerization interface. Four variants (I318T, E340K, I445M, R447G and P453L) increased their mobilities in the C-terminal region, which might act as a semi-mobile tail that is threaded into the dimerization interface. The HDX of most prevalent G194C-hE3 variant was significantly increased at the NAD^+/NADH binding region, which is in consensus with its increased reactive oxygen species generating characteristics. The D444V-hE3 variant induce HDX changes both near the mutation site and around the residue 438, as in the hE3 homodimer D444 and Y438' form a hydrogen bridge. The HDX pattern of K37C-, R460G- and I358T-hE3 variants showed slight perturbations all around the structure.

8. Appling thermofluor method I screened the melting temperatures of hE3 across a 55-conditions. hE3 was characterized by a high thermal stability, the melting temperature in the protein storage puffer was 88.44°C, which was decreased by the high salt concentrations and low pH (54.50°C, 50 mM Na-citrate, pH 5.0 250 mM NaCl). The highest thermal stability (89.50 °C) was observed for buffer: 50 mM PIPES pH 6.7. The

information described here was also useful in combination with the crystallization screens, as the buffer compositions that increase the thermal stability of a protein sample frequently improve the quality of the protein crystals.

9. The buffer composition dependence of the melting temperatures of the pathogenic hE3 variants showed the same overall tendencies as the wild type protein. The hE3 variants still showed high thermal stabilities compared to other human proteins, but the melting temperature of the variants were lower than the hE3, most significant decrease was observed for P453L-hE3 (64.5°C). These results might be explained with a destabilizing effect of the pathogenic mutations.

10. Using the sitting drop method, I crystalized the D444V-hE3 variant. The crystals grew readily within 1-2 weeks and they belonged to the orthorhombic $P2_12_12$ space group and diffracted upto 1.84 Å. The unit cell parameters were =118.04; b=168.94; c=61.28 Å and contained eight protein chains. Dr. Szabó Eszter carried out the detailed analysis of the D444V-hE3 structure.

Conclusions

Applicability of the inclusion complexes of sanguinarine and cucurbit[7]uril. Previous authors showed that the protonation of guest molecules can be prevented by CB7 encapsulation, however, our study was the first example for protection of guest molecules from nucleophilic attack by CB7. Our work also demonstrated that CB7 treatment cause a significant increase in the photostability of SA. CB7 being a biocompatible compound it has not only a potential for stabilizing guest molecules, but also could be used as a drug carrier.

Our paper is cited by structural chemists as being an example for a guest-CBn system, in which the 1:1 and 1:2 complexes are present concurrently and their structures and affinities well characterized. SA is a nearly symmetric molecule: the positively charged nitrogen is located in the center of an aromatic ring system, however, the two ends of SA have slightly different lengths. The CB7 inclusion complexes formed with the two SA ends have more than 3 orders of magnitude difference between the equilibrium constants, which demonstrated the high selectivity of CB7 to its guest molecules.

Insights into the molecular pathomechanism of the hE3-defficiency

We studied the structural background of the hE3 defficieny via solution and crystal structures of the pathogenic hE3 variants. The hydrogen deuterium exchange mass spectrometry experiments revealed the alterations in the solution dynamics caused by the pathogenic mutations of hE3. Dynamical information is analyzed to unveil the role of atomic and molecular motion in the molecular interactions of hE3 in its multienzyme complexes. The insights into mutations modulating molecular dynamics are discussed in relation with enzyme function. The potential structural consequences of the hE3 mutations were to be further investigated in atomic detail by X-ray crystallography. The crystallization methodology described in the dissertation was also useful in elucidating the crystal structures of further pathogenic hE3 variants. The crystal structures of hE3 variants will provide more definitive search models for single molecule electron microscopy, Förster resonance energy transfer or NMR experiments in elucidating transient macromolecular binding complexes.

List of Publications

Publications based on the thesis:

Ambrus, A.*, Wang, J.*, Mizsei, R.*, Zambo, Z., Torocsik, B., Jordan, F., & Adam-Vizi, V. (2016). Structural alterations induced by ten disease-causing mutations of human dihydrolipoamide dehydrogenase analyzed by hydrogen/deuterium-exchange mass spectrometry: Implications for the structural basis of E3 deficiency. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1862*(11), 2098–2109 (2016), DOI: 10.1016/j.bbadis.2016.08.013, IF: 5,476, *first authors

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