Understanding functional dynamics and conformational stability of beta-glycosidases

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CHAPTER *5*

General Conclusion & Perspectives

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Due to their central physiological roles in living organisms, retaining β-glycosidases have been the subject of tremendous research efforts to examine their structure/function relation using numerous biophysical and biochemical approaches. Since the proposition of the hydrolysis mechanism in the late fifties by Koshland[1], the fundamental research on retaining β-glycosidases has been revolutionized by the discovery of multiple reversible and irreversible inhibitors[2-5]. One of the most successful class of inhibitors are mechanism based inactivators, which were extensively used to identify the nucleophilic catalytic residues and to comprehend the catalytic mechanism and substrate itinerary[2, 6, 7]. Subsequently, covalent inhibitors were used as warheads to synthesize chromogenic activity based probes (ABPs), which were widely used to selectively label and discover new retaining β-glycosidases in complex biological samples[8, 9]. For instance, Overkleeft and co-workers have developed ultrasensitive ABPs against human β-glucosidase (GBA)[10]. These ABPs were successfully utilized by Aerts and co-workers to deepen our understanding of the physiopathology underlying Gaucher disease[11, 12]. The organic synthesis and biological applications of these ABPs has become routine[13]. Nevertheless, their binding mechanism and influences on protein conformation and dynamics remained unexplored. Therefore, this work is aimed to establish a bridge between the two research disciplines, using ABP technology to understand functional dynamics and conformational stability of retaining β-glycosidases *in solution* and *in vivo*. The research relied on standard biochemistry and advanced NMR spectroscopy research approaches.

The deficiency of GBA activity in Gaucher patients[14] leads to the accumulation of glucocerebrosides in lysosomes[15] and GM3 gangliosides in plasma[16]. Such accumulations are the cause of the clinical manifestations of the disease[17, 18]. Continuous intravenous administration of exogenous GBA (rGBA) is, so far, the most efficient treatment for Gaucher disease[19-24]. It remains costly[25] and non-affordable for many patients around the world, especially in countries with low health care quality. Thus, finding an inexpensive therapeutic alternative is imperative.

The endo-glycosylceramidase II from *Rohodococcus* sp. is a bacterial homologue of GBA able to endo-hydrolyse the glycosidic link in complex glycosylceramidases. The enzyme was first discovered in 1980s by Ito et al. and found multiple applications in gangliosides analysis[26]. The crystal structure of EGCII was solved in 2007 by Strynadka and co-workers in the free and GM3 ganglioside-bound forms.[27] In theory, this protein holds promise as a potential drug against Gaucher disease and other glycosphingolipidose disorders, *via* administration once in a lifetime for clearance of the accumulated gangliosides. Hence, ABPs were used to gain insight into factors that influence the conformational stability of EGCII as a preliminary step toward its design as a therapeutic agent. The study revealed that the protein is thermolabile and highly flexible in its free state. The inherent flexibility of EGCII is postulated to be requested for its peripheral

interaction with the cell membrane to exert its function against its amphiphilic substrates. This hypothesis was supported by the highly exposed hydrophobic surface to the solvent probed by ANSA. Flexibility could also play a role in the interaction between EGCII and its activator, a protein partner that enhances its activity against gangliosides in the absence of detergents[28]. Upon complex formation with hydrophilic, activity-based inhibitors, a minor increase in thermostability and rigidity were observed, concomitant with a preserved high hydrophobic surface exposed to the solvent. However, the interactions with activity-based inhibitors with high lipophilic potential increased the protein thermostability and rigidity, and reduced its exposed hydrophobic surface. The stabilization of EGCII conformation correlates with the shape and hydrophobicity of the substituents of the ABPs. It was concluded that amphiphilic active site binders that mimic the natural substrate are superior stabilizers, compared to their small hydrophilic counterpart. By virtue of ABPs technology, the characteristics of the enzyme-inhibitors complex were investigated and helped to elucidate which parts of the inhibitors are important for efficient stabilization activity. This information could be further exploited in the design of more specific ABPs and inhibitors against other β-glycosidases. EGCII thermolability and flexibility might hamper its therapeutic use, and, therefore, one strategy to overcome these disadvantages is to re-engineer the protein by including glycosylation sites on its surface[29]. This approach might enhance EGCII stability and also assist its uptake by macrophages receptors through those glycosylation sites[20].
Additionally, an endoglycoceramidase activity towards gangliosides has been reported for human cancer cells[30], but the protein responsible for such activity has not been characterized yet. The development of endo-glycosidases activity-based probes (endo-ABPs) could help in uncovering the enzyme behind the endo-hydrolysis of gangliosides in cancer cells and their role in cancer pathology. If the endoglycoceramidase is a marker for cancer cells phenotype, ultrasensitive endo-ABPs might be also be used for diagnosis purposes in the initial stage of cancer development.

In addition to enzyme replacement therapy in Gaucher disease, new therapeutic approaches are under intense investigation, based on identifying small chemical molecules that rescue GBA proteostasis in Gaucher patients[31-34]. Those small molecules called "pharmacological chaperones" are supposed to stabilize GBA mutants fold in the ER compartment of the cells, thus escaping the quality control machinery in the ER compartment, so that they can reach their final lysosomal destination[31, 35]. Several GBA active site binders have validated this proof of principle *in vivo* and *in vitro*[36-39]. However, their stabilization mechanism on GBA and their effects on the enzyme conformation in solution remained unclear. Therefore, reversible, semi-reversible (time dependent) and irreversible (ABPs) inhibitors with different chemical characteristics were used to investigate the stabilization mechanism of those active site binders on GBA fold *in vitro* and *in vivo*.

The outcomes of the study revealed that small active site binders, which only occupy the glycon pocket of the active site, provoke minor changes of the biophysical properties of the protein. When interacting with amphiphilic ABPs, which are thought to occupy simultaneously the glycon and aglycon sites, a remarkable increase of the protein thermal stability was observed (up to 21°C) in conjunction to its resistance against trypsin digestion, suggesting rigidification. Additionally, ABPs induced a shift of the GBA fluorescence spectrum toward the blue region, again suggesting a more compact conformation upon complex formation. Serendipitously, an iFRET mechanism between the BODIPY fluorescence reporter of the ABPs and the protein tryptophan residues was observed. This phenomenon could potentially be exploited as a method for cell imaging. By exciting the indole rings of GBA tryptophans and recording the BODIPY emission spectrum of the ABPs, the background signals originating from free ABPs might be bypassed and clearer images could be obtained[40].

The *in vitro* findings were validated *in vivo* by observing a correlation between the binding site occupancy of GBA by ABPs and the intracellular increase of the protein level.

The studies on EGCII and GBA lead to the conclusion that both proteins share a similar stabilizing mechanism by active site binders. Therefore, evidence is provided about the increase in cellular levels of GBA by ABPs and reversible inhibitor is in part caused by their ability to stabilize GBA folding, which increases its resistance against breakdown by lysosomal proteases. Those finding provided extra information that could be considered when designing new pharmacological chaperones for Gaucher disease.

Despite the devoted efforts to discover an optimal candidate for pharmacological chaperone treatment, none of the available compounds has reached the clinic. Isofagomine was the most successful compound but failed to pass the clinical trials, presumably due to its poor biodistribution, ascribed to its hydrophilicity[41]. Therefore, a hybrid therapy between pharmacological chaperones and enzyme administration represent a therapeutic alternative, which is under investigations. Continuous administration of rGBA to Gaucher patients is required because of the short half-life in the blood[42]. Providing a cocktail of rGBA and isofagomine might increase the half-life of the protein under physiological conditions, thus lowering the frequency of the enzyme transfusion and the cost of the treatment. However, this approach might require a high dose of isofagomine to warrant an effective rGBA stabilisation that could induce unwanted side-effects.

An alternative in this hybrid therapy is the use of a fluorinated glucoside inhibitor, instead of isofagomine. This class of inhibitors is supposed to bind rGBA in a time dependent manner[43, 44]. The fluoride in the C2 position of the pyranose ring ensures slow dissociation of the enzyme-inhibitor complex, providing sufficient time for rGBA to reach its destination without being degraded in the blood circulation. The one to one ratio between the enzyme-inhibitor cocktail could reduce the possible side effects, compared to isofagomine.

The use of GBA active site binders as pharmacological chaperones appears promising, however, their potential inhibition of the enzyme activity at high dose represents a potential problem. Therefore, attention should also be paid to allosteric pharmacological chaperone because they bind remote from the active site, avoiding such inhibitory effects.

Like EGCII, GBA also has an activator called saposin C[45]. Saposin C is an 80-amino acid protein and its structure was determined by NMR spectroscopy and X-ray crystallography under different condition[46-48]. Saposin C binding to GBA induces significant changes of its fluorescence spectrum, suggesting changes in the GBA conformational state upon protein-protein complex formation[49]. Attempts to characterize the GBA-saposin C complex using X-ray crystallography was unsuccessful. The complex was modelled by molecular docking though[50]. Uncovering the saposin C binding site on GBA experimentally might give an idea about which structural location should be targeted to design allosteric pharmacological chaperones. The binding site could be studied by paramagnetic NMR spectroscopy. A new type of activity-based probes against GBA could be developed for this purpose, using epoxide as a warhead linked to lanthanoid paramagnetic NMR tag (PABP) instead of BODIPY. The covalent complex between GBA and the PABP might be used to obtain experimental restraints (PREs, PCSs and RDCs) of the GBA-Saposin C complex[51-54]. The experimental restraints can be used to direct the docking between the two proteins, providing realistic model of their complex[55]. Uncovering the saposin C binding site on GBA surface will assist the design of allosteric pharmacological chaperone.

The characteristic shared between the different families of retaining β-glycosidase is their conserved and well understood catalytic mechanism. This has somehow limited the research devoted to understand the contribution of the enzyme dynamics and conformational changes in the different steps of the catalytic reaction. In the present work, this question was addressed using BCX as a model. the choice was based on the suitable biophysical characteristics of BCX for NMR spectroscopy studies. Moreover, this protein has been extensively investigated by NMR spectroscopy and X-ray crystallography in combination with other biochemistry techniques[56-62].This knowledge provided a solid starting point that facilitated the task in exploring the conformational landscape and dynamics of this protein during its catalytic pathway. By incubating the protein with different non-covalent ligands and a covalent active based inhibitor, a novel compound that was specifically made for this study[63], the enzyme-ligand complexes were systematically characterized in every step of the catalytic reaction using paramagnetic NMR spectroscopy and CPMG RD techniques. In its free state, the protein populates a ground state similar to its crystalline state as concluded from PCS data. The protein displays subtle millisecond dynamics in the thumb and fingers regions with an excited state population = 0.7%. The Michaels complex displays an induced fit

mechanism as shown by CSP analysis. This observation has not been reported before for a GH11 xylanase. Both states of the enzyme appear to have different conformations compared to the free protein, as concluded from the paramagnetic NMR data. The exchange rate between the initial and induced states is low, on the order of the turn-over rate of the enzyme, suggesting that the formation of the induced state may contributed to the rate limiting step of catalysis. In the ES state, the millisecond dynamics is strongly enhanced with a population of the excited state(s) of 9%. This finding is in accordance with a previous report on another GH11 xylanase family member. No major changes of the protein dynamics behaviour were observed in the case of the EI state compared to the free protein, despite the occurrence of conformational changes upon its covalent modification by epoxyX2. In the EP state the protein displays minor conformational changes upon ligand binding, again concomitant with enhancement of the millisecond dynamics, similar to the ES state. The ES and the EP ground states are connected *via* excited states to the EI ground state conformation. Based on those findings, a description of BCX conformational landscape and dynamics during catalysis is proposed, providing evidence for involvement of enzyme motions in the catalytic mechanism.

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