Structural basis of C1-inhibitor specificity and deficiency

Doctoral (PhD) thesis summary

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INTRODUCTION

C1-inhibitor (C1-inh) is the principal regulator of the complement system and bradykinin releasing system in human blood. As a serpin type protease inhibitor of these cascades, C1-inh has wide, yet specific inhibitory spectrum. Owing to this C1-inh has anti inflammatory activity. My goal was to understand the mechanism that explains how can a single inhibitor achieve such versatility.

It is a long known observation that the serpins (serine protease inhibitors, resembling "mousetraps") are inherently non-specific (Gettins & Olson, J. Biol. Chem., 2009). This is a consequence of the highly flexible substrate-like reactive center loop (the "bait"), and of the irreversible trapping mechanism (the "mousetrap"). After "bait" cleavage, the protease reactive center becomes distorted, and the catalysis is frozen at the covalent acyl-enzyme intermediate step. This energetically unfavorable process seems to be compensated by the serpin reactive center loop insertion, during which the surface "bait" becomes a stable strand in a serpin β-sheet. It seems that any protease can be inhibited if there is a covalent intermediate during catalysis. Since C1-inh contains an arginine at the P1 site, it is now less surprising that C1-inh was observed to inhibit essentially all proteases that have preference for cleaving at basic residues. Such proteases are quite abundant in the compartment where C1-inh resides (from thrombin to plasmin, fXIa, fXIIa, plasma kallikrein, MASP-1, MASP-1, MASP-2, C1r, C1s, …). Is it possible that C1-inh relies solely on the sequence of its reactive center loop to modulate specificity?

A clue that C1-inh may employ a more elaborate mechanism than that comes from the observations that it is modulated by the acidic polysaccharide heparin. Modulation ranges from slight "inhibition of inhibition" (0.5-fold slowed reaction velocity against contact activation protease fXIIa) to a strong potentiation (two magnitude acceleration against coagulation protease fXIa and to a lesser extent complement C1s). If we focus on other serpins, we find amazingly sophisticated mechanisms that not only ensure specificity towards proteases, but allow for modulating it. The prototype of a such mechanism is the modulation of antithrombin activity by heparin. *In vivo* the heparin (and heparan) chains line the wall of blood vessels. Antithrombin bound to a specific pentasaccharide unit in heparin chains becomes allosterically activated by expelling its reactive center loop. The flexible loop protruding from the body of protein is now more accessible for proteases. In itself this still cannot account for the effect of heparin. In addition, thrombin also binds the same heparin chain, but with lower affinity, so it migrates along the chain, until finding the antithrombin molecule. Thus, heparin serves as a bridge

between serpin and protease and stabilizes the encounter complex ("bridging" mechanism). The combination of these events results in an incredible 10,000-fold acceleration of the reaction, efficiently stopping any clot formation.

C1-inh was studied by many since its first identification. Probably the studies on C1-inh deficiency show best the importance and the large size of the field. The C1-inh deficiency is a hideous, but potentially lethal disease. Since C1-inh is an anti-inflammatory molecule, its application is straightforward in therapies. However, the studies so far were missing an aspect of C1-inh biology: the questions and phenomenons were not explained at atomic level. I knew that several attempts were made over the years (or perhaps over the decades) to solve the structure of C1-inh. I set out to tackle this unsolved problem in my doctoral study.

GOALS

Since the first serpin structure was published (Loebermann *et al.*, J. Mol. Biol., 1984) a great deal of information was gathered about serpins, about their mode of action and their role in health and disease. What is lacking behind is the knowledge on specific mechanisms that govern targeting of individual serpins to their proteases in the dense environment of blood (e.g. the "bridging" mechanism). The core serpin domain fold seems static and highly conserved. However, the majority of serpins have "additions" to this core, including glycosylation (C1-inh), disulfide switches (plasminogen activator inhibitor-2), N- or C- terminal polypeptide extensions (heparin cofactor II, C1-inh, α_2-antiplasmin). How these "surplus" elements work, what additional mechanisms regulate the biological response from serpins is of high importance. The successes with therapies exploiting serpins (e.g. antithrombin, C1-inh) suggest that research in this direction can yield medically important discoveries. In my doctoral work I focused on C1-inh, a less-well studied member of serpins compared to antithrombin or α_1-protease inhibitor. My specific questions regarding C1-inh can be summarized as follows:

1. What are the accessory mechanism(s) that regulate C1-inh activity against proteases?
2. How does heparin change the activity of C1-inh?
    a. Is it similar to the heparin acceleration of antithrombin?
    b. Why is heparin together with C1-inh rate-enhancing against some proteases (fXIa, C1s) while against others has no (plasma kallikrein) or reverse effect (fXIIa)?
3. What is the role of the ~100 amino acid long N-terminal extension and its extensive glycosylation?

I planned to solve the crystal structure of C1-inh in order to answer these questions.

METHODS

- Recombinant DNA technology

  Truncated and mutant constructs of C1-inh were designed and cloned. Customized vectors were used for the expression and cloning.

- Recombinant protein expression

  The designed C1-inh constructs were expressed in *E. coli* and *P. pastoris*, using both shake-flasks and fermentor. The *E. coli* derived protein was refolded. The *P. pastoris* fermentation process was optimized for expression.

- Protein purification and crystallization

  The recombinant C1-inh from *P. pastoris* was purified using Ni^{2+}-affinity chromatography and other chromatography. The protein was deglycosylated enzymatically with $EndoH_f$ glycosidase and purified. A C1-inh form was crystallized using the method of hanging drops.

- X-ray crystallography, modelling and other computations

  The structure of C1-inh was solved using X-ray crystallography with the method of molecular replacement. The electrostatic potential surfaces, docking of a heparin disaccharide, and other calculations were perfomed computationally.

- Miscellaneous measurements

  The activities and behaviour of C1-inh against proteases were tested using SDS-PAGE. Thermal stability was measured using DSC, heparin affinity was probed with heparin affinity chromatography.

RESULTS

1. Different C1-inh forms were expressed in different expression systems, and the expression conditions were successfully optimized.

2. A novel, so far uncharacterized inactive (latent) form of C1-inh was described.

3. The glycosylation of C1-inh is extensive, which prevents crystallization. A gentle enzymatic method was developed to solve this problem.

4. The crystal structure of latent C1-inh serpin domain was solved at 2.4 Å resolution. In contrast, the N-terminal domain is likely disordered.

5. Atomic level explanation of C1-inh deficiency is given.

6. A simple mechanism ("sandwich-mechanism") was proposed to explain the effect of heparin.

CONCLUSIONS

In structural biology the "glycosylation problem" can be solved by expressing protein in hosts that produce Endo H sensitive glycans. After expression, the glycans can be removed using Endo H. The variants of this method became popular recently independently of my results (Chang *et al.*, Structure, 2007).

The structure immediately offered an explanation of inactivity and why many naturally occurring mutations yield latent and polymeric C1-inh forms that cause disease. For example, a subset of mutations (e.g. Ala436Thr) likely introduce hydrogen bonds in the structure that make the latent form more stable over the native one. In contrast, other mutations (e.g. Pro476Ser) perhaps decrease the energy barrier during the active-latent transition. My report was the first on the plasticity of the residues after helix I in the otherwise highly conserved fold of serpins. Now it is known that such plasticity is an essential prerequisite of serpin polymerization, because it allows steric clash free propagation of linear polymer chains (Yamasaki *et al.*, Nature, 2008).

A simple "sandwich" mechanism is proposed which fits best with available data. Briefly, the heparin molecules insert between serpin and protease in the rate-determining encounter complex. The negatively charged heparin counteracts the repulsive interaction between positively charged C1-inh and protease, and explains why heparin is rate-enhancing only with positively charged proteases (C1s and fXIa), and neutral or rate-decreasing on the neutral plasma kallikrein and acidic fXIIa.

Ultimately the results described in this thesis contribute to our molecular level understanding of hereditary angioedema, the disease caused by C1-inh deficiency. Since C1-inh is an already used, clinically important molecule, it seems possible to engineer recombinant C1-inh with enhanced activity to be used in therapies (Beinrohr *et al.*, Trends Mol. Med., 2008).

PUBLICATIONS RELATED TO THIS THESIS

The presenting author at conferences is indicated in **bold**.

Publications in refereed scientific journals:

1. László Beinrohr, József Dobó, Péter Závodszky, Péter Gál
   "C1, MBL-MASPs and C1-inhibitor: novel approaches for targeting complement-mediated inflammation"
   Trends Mol. Med. (2008) 14: 511-521

2. László Beinrohr, Veronika Harmat, József Dobó, Zsolt Lörincz, Péter Gál, Péter Závodszky
   "C1 Inhibitor Serpin Domain Structure Reveals the Likely Mechanism of Heparin Potentiation and Conformational Disease"
   J. Biol. Chem. (2007) 270: 21100-21109

Abstracts in refereed scientific journals:

3. Veronika Harmat, László Beinrohr, József Dobó, Zsolt Lőrincz, Péter Gál, Gábor Náray-Szabó, Péter Závodszky
   "C1-inhibitor structure reveals a novel mechanism of heparin potentiation"
   Acta Crystallogr. A (2007) 63: s129

4. László Beinrohr, Veronika Harmat, József Dobó, Zsolt Lőrincz, Péter Gál, Péter Závodszky
   "Crystal structure of C1-inhibitor: Understanding the mechanism of its deficiency and heparin's antiinflammatory activity"
   Mol. Immunol. (2007) 44: 3928

Conference presentations:

5. **László Beinrohr**, Veronika Harmat, József Dobó, Zsolt Lőrincz, Péter Gál, Péter Závodszky
   "A komplement C1-inhibitor térszerkezete - amit megtudtunk a C1-inhibitor heparin aktiválásáról és deficienciájáról"
   2007, Hajdúszoboszló, a Magyar Immunológiai Társaság XXXVI. Vándorgyűlése

6. **László Beinrohr**, Veronika Harmat, József Dobó, Zsolt Lőrincz, Péter Gál, Péter Závodszky
   "Crystal structure of C1-inhibitor: understanding the mechanism of its deficiency and heparin's antiinflammatory activity"
   2007, Cardiff, 11th European Meeting on Complement in Human Disease

7. **László Beinrohr**, Veronika Harmat, József Dobó, Zsolt Lőrincz, Péter Gál, Péter Závodszky
   "A C1-inhibitor térszerkezete: a polianionok moduláló hatásának és egy konformációs betegség mechanizmusának atomi szitű magyarázata"
   2007, Debrecen, a Magyar Biokémiai Egyesület Vándorgyűlése

8. **Péter Gál**, László Beinrohr, József Dobó, Veronika Harmat, Zsolt Lőrincz, Péter Gál, Péter Závodszky
   "Crystal structure of C1-inhibitor: insight into the mechanism of conformational disease"
   2007, Budapest, 5th C1 Inhibitor Deficiency Workshop

9. **László Beinrohr**, Veronika Harmat, József Dobó, Zsolt Lőrincz, Péter Gál, Péter Závodszky
   "Crystal structure reveals how polyanions bind and regulate activity of the multifunctional regulatory protein, C1-inhibitor"
   2006, Szeged, Straub-napok

10. **Zsolt Lőrincz**, László Beinrohr, Péter Závodszky, Péter Gál
    "HUMÁN REKOMBINÁNS C1-INHIBITOR ELŐÁLLÍTÁSA BAKTÉRIUMOKBAN"
    2004, Sopron, a Magyar Biokémiai Egyesület Vándorgyűlése

Conference posters:

11. **László Beinrohr**, Veronika Harmat, József Dobó, Zsolt Lőrincz, Péter Gál, Péter Závodszky
    "Crystal structure of C1-inhibitor: insight into the mechanism of conformational disease and heparin modulation of inflammation"
    2008, Leuven, Serpins2008

12. **László Beinrohr**, Veronika Harmat, József Dobó, Zsolt Lőrincz, Péter Gál, Péter Závodszky
    "Crystal structure of C1-inhibitor: understanding the mechanism of its deficiency and heparin's antiinflammatory activity"
    2007, Cardiff, 11th European Meeting on Complement in Human Disease

13. **Veronika Harmat**, László Beinrohr, József Dobó, Zsolt Lőrincz, Péter Gál, Gábor Náray-Szabó, Péter Závodszky
    "C1-inhibitor structure reveals a novel mechanism of heparin potentiation"
    2007, Marrakech, 24th European Crystallographic Meeting

14. **László Beinrohr**, József Dobó, Veronika Harmat, Zsolt Lőrincz, Péter Gál, Péter Závodszky
    "Crystal structure of C1-inhibitor: understanding the mechanism of heparin potentiation"
    2007, Budapest, 5th C1 Inhibitor Deficiency Workshop

15. **László Beinrohr**, Zsolt Lőrincz, Péter Gál, Péter Závodszky
    "Production of human recombinant C1-inhibitor in Escherichia coli"
    2004, Szeged, Straub-napok