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REGULAR ARTICLE

Investigating serpin—enzyme complex formation and stability via single and multiple residue reactive centre loop substitutions in heparin cofactor II☆

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Abstract

Introduction: Following thrombin cleavage of the reactive centre (P1—P1′; L444—S445) of the serpin heparin cofactor II (HCII), HCII traps thrombin (IIa) in a stable inhibitory complex. To compare HCII to other serpins we substituted: the P13—P5′ residues of HCII with those of α_1-proteinase inhibitor (α_1-PI), α_1-PI (M358R), or antithrombin (AT); the P4—P1, P3—P1, and P2—P1 residues of HCII with those of AT; and made L444A/H/K/M or R point mutations. We also combined L444R with changes in the glycosaminoglycan binding domain collectively termed MutD.
Materials and methods: Variants were made by site-directed mutagenesis, expressed in bacteria, purified and characterized electrophoretically and kinetically.
Results and conclusions: Of the P13—P5′ mutants, only the α_1-PI-loop variant retained anti-IIa activity, but less than the corresponding L444M. Heparin-catalyzed rate constants for IIa inhibition were reduced vs. wild-type (WT) by at most three-fold for all P1 mutants save L444A (reduced 20-fold). L444R and L444K inhibited IIa>50- and >6-fold more rapidly than WT in heparin-free reactions, but stoichiometries of inhibition were increased for all variants. HCII—IIa complexes of all P1 variants were stable in the absence of heparin, but those of the L444K and

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L444R variants released active IIa over time with heparin. Limited proteolysis of these two groups of HCII—IIa complexes produced different fragmentation patterns consistent with conformational differences. The combination of either substituted AT residues at P2, P3, and P4, or the MutD mutations with L444R resulted in complex instability with or without heparin. This is the first description of HCII—IIa complexes of transient stability forming in the absence of heparin, and may explain the extent to which the reactive centre loop of HCII differs from that of AT.

The plasma protein heparin cofactor II (HCII) belongs to the serine protease inhibitor (serpin) family of proteins [1]. HCII is a 480 amino acid residue glycoprotein with a molecular mass of 66 kDa [2]. It regulates coagulation by inhibiting thrombin in a reaction catalyzed by glycosaminoglycans (GAGs) such as heparin [2]. Of the heparin-binding serpins, HCII is unique in that its activity is enhanced by dermatan sulphate, a predominantly extravascular GAG [3] that can also be found in plasma during pregnancy [4]. While HCII—thrombin complexes are found in plasma, inherited HCII deficiency does not appear to increase the risk of thrombosis in patients [5,6], leaving its physiological role uncertain. However, mice made deficient in HCII by targeted gene disruption are less resistant to photochemically-induced arterial thrombosis than normal controls [7], suggesting an antithrombotic function of the serpin. Similarly, high plasma HCII levels have recently been shown to be protective in patients undergoing stenting for either coronary [8] or peripheral arterial disease [9].

HCII is frequently compared to another serpin, antithrombin (AT), with which it shares an amino acid sequence homology of approximately 30%, a similar three-dimensional structure [10] and GAG-binding ability. Nevertheless, the two serpins differ in target protease and GAG specificity. While AT is able to inhibit many proteases involved in coagulation, including thrombin and factors Xa and IXa, HCII specifically inhibits thrombin [11]. The rate of inhibition of IIa by HCII is increased 1000-fold in the presence of either heparin [2] or dermatan sulphate, whereas inhibition of thrombin and factor Xa by AT is accelerated by heparin alone [12,13]. HCII also possesses an N-terminal domain rich in acidic residues, one that has no counterpart in AT, and which has been shown to mediate HCII binding to thrombin exosite I following allosteric activation of HCII by heparin [14—16]. A heparin bridge between HCII and thrombin exosite II also makes a minor contribution to the catalysis of thrombin inhibition by this serpin [17]. In contrast, thrombin inhibition by AT in the presence of full-length heparin depends predominantly on the formation of a heparin bridge between AT and exosite II [18], although it has recently been suggested that aspects of the initial binding of heparin to both serpins are similar [19].

Structurally, serpins are characterized by a reactive centre loop (RCL) that protrudes from the serpin body, which consists of three β-sheets and eight or nine α-helices [1,20]. A scissile bond in the RCL referred to as P1—P1′ (according to Schechter and Berger's nomenclature [21]) is attacked by a cognate protease, resulting in the release of stored energy, the insertion of the RCL into β-sheet A, and translocation of the covalently attached protease to the opposite pole of the serpin [22]. Full translocation of the attacking protease has been suggested to be accompanied by its compression against the body of the serpin, causing a distortion of the protease active site that prevents or greatly slows the release of active protease from the serpin—enzyme complex [23]. The result is a stable 1:1 covalent complex between the serpin and protease [22,23].

While the structure of a serpin RCL is clearly tightly linked to both the efficiency and specificity of its inhibition of proteases, it has been difficult to discern the rules governing these processes by either P1 mutagenesis studies or more extensive loop exchanges. In the Pittsburgh variant of α_1-proteinase inhibitor (α_1-PI), substitution of the WT P1 Met by Arg redirected α_1-PI away from its natural target protease, neutrophil elastase, and instead conferred substantial inhibitory activity against thrombin, factor Xa, factor XIa, kallikrein, plasmin [24] and activated protein C [25]. In contrast, mutation of the P1 residue of α_1-antichymotrypsin (α_1-AC) from Leu to Met was insufficient to convert the serpin into an effective elastase inhibitor; the second-order rate constant of the mutant α_1-AC was found to be three orders of magnitude less than the wild-type [26]. Similarly, the substitution of AT P19—P2′ residues in PAI-1 did not redirect this serpin towards thrombin as a target protease [27] and an HCII P16—P3′ substitution in α_1-PI did not yield a functional inhibitor [28].