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The M358R variant of α_1-proteinase inhibitor inhibits coagulation factor VIIa

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ABSTRACT

The naturally occurring M358R mutation of the plasma serpin α_1-proteinase inhibitor (API) changes both its cleavable reactive centre bond to Arg–Ser and the efficacy with which it inhibits different proteases, reducing the rate of inhibition of neutrophil elastase, and enhancing that of thrombin, factor XIa, and kallikrein, by several orders of magnitude. Although another plasma serpin with an Arg–Ser reactive centre, antithrombin (AT), has been shown to inhibit factor VIIa (FVIIa), no published data are available with respect to FVIIa inhibition by API M358R.

Recombinant bacterially-expressed API M358R and plasma-derived AT were therefore compared using gel-based and kinetic assays of FVIIa integrity and activity.

Under pseudo-first order conditions of excess serpin over protease, both AT and API M358R formed denaturation-resistant inhibitory complexes with FVIIa in reactions accelerated by TF; AT, but not API M358R, also required heparin for maximal activity. The second order rate constant for heparin-independent API M358R-mediated FVIIa inhibition was determined to be $7.8 \pm 0.8 \times 10^2$ $M^{-1}sec^{-1}$.

We conclude that API M358R inhibits FVIIa by forming inhibitory complexes of the serpin type more rapidly than AT in the absence of heparin. The likely 20-fold excess of API M358R over AT in patient plasma during inflammation raises the possibility that it could contribute to the hemorrhagic tendencies manifested by rare individuals expressing this mutant serpin.

1. Introduction

Members of the serpin superfamily such as α_1-proteinase inhibitor (API) form stable, covalently bonded inhibitory complexes following target protease attack on the serpin reactive centre bond [1]. The naturally occurring API M358R variant (also called API-Pittsburgh) demonstrates sharp alterations in inhibitory specificity due to conversion of its reactive centre bond from Met–Ser to Arg–Ser [2]. The mutant protein inhibits the preferred physiological target of API, neutrophil elastase, at rates three to four orders of magnitude slower than the wild-type protein [2], while demonstrating commensurately increased inhibition rates for other proteases such as thrombin [2], factor Xa [3], factor XIa, factor XIIa, kallikrein [4], and activated protein C [5]. This altered inhibitory profile explains in part the bleeding tendencies of members of three unrelated families who have been found to carry this alteration of codon 358 of the API gene [2,6,7]. Two groups reported in 1993 that another serpin, antithrombin (AT), which, like API M358R, contains an Arg–Ser (Arg393-Ser394) reactive centre, inhibits coagulation factor VIIa (FVIIa) [8,9]. Because API M358R is, like AT, a relatively promiscuous protease inhibitor, we hypothesized that it would inhibit FVIIa, a reaction not previously described in the biomedical literature. We report here that API M358R, unlike its wild-type counterpart, inhibits FVIIa in a reaction accelerated by tissue factor (TF) but unaffected by heparin.

2. Materials and methods

2.1. Proteins

Plasma-derived purified AT and recombinant full-length TF were purchased from Hematologic Technologies (Essex Junction,

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VT, USA). Recombinant FVIIa (Niastase) was from Novo Nordisk, (Mississauga, Canada). Recombinant API M358R was expressed in N-terminally His_6-tagged form under arabinose control in *E. coli* TOP10 cells (Thermo-Fisher Scientific, Waltham, MA, USA) and purified to homogeneity using nickel-chelate and ion exchange chromatography as previously described [10].

2.2. Gel-based analysis of FVIIa inhibition

Recombinant FVIIa was reacted with purified serpins (either AT or API M358R) in 25 mM HEPES pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$in the presence or absence of TF or 10 U/ml heparin (sodium salt from porcine intestinal mucosa, Grade 1A, ≥ 180 US Pharmacopeia units/mg, Sigma–Aldrich, St. Louis, MO, USA) at 37 °C. At timed intervals aliquots were removed, quenched in SDS-PAGE sample buffer, and analyzed on 10% SDS-polyacrylamide gels stained after electrophoresis with Coomassie Brilliant Blue.

2.3. Kinetic comparison of FVIIa inhibition by AT or API M358R

Inhibition of FVIIa-mediated amidolysis was monitored kinetically using chromogenic substrate S2288 (DiaPharma, West Chester, OH, USA) in discontinuous assays. Pseudo-first order conditions of excess serpin (500 nM AT or API M358R), 25 nM FVIIa, 25 nM TF in 25 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, 0.1% (w/vol) BSA (HEPES reaction buffer, HRB) at 37 °C were employed. Some reactions were supplemented with 1.0 U/ml heparin. At timed intervals, reaction aliquots were diluted 1:10 into HRB containing 1.0 mM S2288, and the residual FVIIa activity was determined by monitoring the change in absorbance at 405 nm using a plate reader.

2.4. Determination of the second order rate constant of FVIIa inhibition

Second order rate constants (k_2) were determined for the reaction of 500 nM API M358R with 25 nM FVIIa/25 nM TF in HRB, with or without supplementation with 1.0 U/ml heparin, by dividing the pseudo-first order rate constant k_1 by the API M358R initial concentration. Values for k_1 were obtained by determining the slope of the plot of the natural logarithm of the ratio uninhibited FVIIa amidolysis to its inhibited counterpart after t seconds of inhibition by API M358R versus time, analogously to our previous approach to k_2 determination of thrombin inhibition [10].

2.5. Statistical methods

Statistical comparisons were made with the aid of GraphPad Instat software (version 3.1, GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

We first confirmed AT-mediated inhibition of FVIIa using gel-based assays (Fig. 1). As expected based on earlier reports [8,9], incubation of equimolar AT with FVIIa in the presence of heparin resulted in the formation of an SDS-stable inhibitory complex. Complex formation was promoted in the presence of TF (Fig. 1, compare third and fourth lanes). Under reducing conditions it was clear that the heavy chain, which contains the FVIIa catalytic site, was partially consumed in forming the denaturation-resistant inhibitory complexes, as expected for a serpin-enzyme reaction product in which the N-terminal amino acid of the reactive centre and the active site serine of the protease are linked by an acyl ester bond [1]. The differential Coomassie Blue staining intensity of the FVIIa light chain relative to the heavy chain has been previously noted [11].

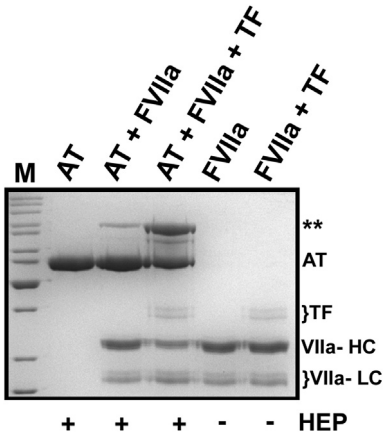

Fig. 1. Formation of SDS-stable complexes between AT and FVIIa. AT (5 μM), FVIIa (5 μM) and TF (1 μM) were combined in the presence of 10 U/ml heparin (HEP) for 5 min at 37 °C (AT + FVIIa + TF, 4th lane), and compared to reactions lacking one (AT + FVIIa) or two (AT) protein reactants or mixtures of FVIIa and TF (FVIIa + TF) or TF alone. A 12% SDS-polyacrylamide gel electrophoresed under reducing conditions and stained with Coomassie Blue is shown. M refers to molecular mass markers of 200, 150, 120, 100, 85, 70, 60, 50 (darker intensity), 40, 30, and 25 kDa. The position of AT-FVIIa complexes (**), AT, TF, and the heavy (FVIIa-HC) and light (FVIIa-LC) chains of FVIIa are indicated, at right. Equivalent portions of reactions or control mixtures were electrophoresed in each lane.

Serpin–FVIIa reactions were next analyzed under more physiologically likely conditions of excess serpin (Fig. 2). In the absence of TF, incubation of API M358R with FVIIa led to the slow formation of a 75 kDa AT-FVIIa heavy chain complex; addition of TF resulted in greater conversion of FVIIa into inhibitory complexes in 0.5–1.0 min than was observed in 30–60 min in its absence (compare Fig. 2A to B). A similar stimulatory effect of TF was noted for AT reactions, although heparin was an obligatory cofactor under these conditions (compare Fig. 2C to D). These observations were borne out by more quantitative assays employing amidolysis of a chromogenic substrate; the API M358R-FVIIa reaction appeared insensitive to heparin, while inhibition by AT under these conditions was completely heparin-dependent (Fig. 3, compare diamond to inverted triangle progress curves). Indeed, k_2 values for FVIIa inhibition by API M358R were unaffected by the presence ($7 \pm 2 \times 10^2$ $M^{-1}sec^{-1}$) or absence of heparin ($7.8 \pm 0.8 \times 10^2$ $M^{-1}sec^{-1}$) ($n = 7–10$, $p = 0.32$, non-significant by two tailed unpaired t test).

API M358R was found to inhibit FVIIa at a much slower rate than other serine proteases, even when the conformation of FVIIa was optimized by TF binding. Using API M358R produced in the same bacterial expression system as in the current study, we previously reported mean rate constants for the inhibition of thrombin, factor Xa, factor XIa, and factor XIIa of 4.7, 0.41, 2.6, and 0.26×10^5 $M^{-1}sec^{-1}$ [12], indicative of inhibition rates 33- to 600-fold faster than that observed for FVIIa. Rates of AT-mediated inhibition of FVIIa similarly fall short of those mediated by this serpin on other proteases; in the absence of heparin, reported k_2 values for AT-mediated FVIIa/TF inhibition only range from 0.33 [13] to 4.5×10^2 $M^{-1}sec^{-1}$ [8]. Heparin catalyzes a modest acceleration of inhibition of FVIIa by AT, into the range of 0.56 [8] to 1.5×10^4 $M^{-1}sec^{-1}$ [13], far less than the >4000-fold enhancement it confers on AT-mediated inhibition of thrombin [14]. Binding of heparin accelerates protease inhibition by some serpins via conformational changes transmitted to the reactive centre loop, via template effects if the protease also binds heparin, or via both mechanisms [1,15]. Since both AT and FVIIa bind heparin [16], some of the inhibition of FVIIa likely involves a template mechanism