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BRIEF REPORT

WEDGE: an anticoagulant thrombin mutant produced by autoactivation

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Summary. *Background:* The production of therapeutically relevant proteases typically involves activation of a zymogen precursor by external enzymes, which may raise regulatory issues about availability and purity. Recent studies of thrombin precursors have shown how to engineer constructs that spontaneously convert to the mature protease by autoactivation, without the need for external enzymes. *Objectives:* Autoactivation is an innovative strategy that promises to simplify the production of proteases of therapeutic relevance, but has not been tested in practical applications. The aim of this study was to provide a direct test of this strategy. *Methods:* An autoactivating version of the thrombin mutant W215A/E217A (WE), which is currently in preclinical development as an anticoagulant, was engineered. *Results and Conclusions:* The autoactivating version of WE can be produced in large quantities, like WE made in BHK cells or *Escherichia coli*, and retains all significant functional properties *in vitro* and *in vivo*. The results serve as proof of principle that autoactivation is an innovative and effective strategy for the production of trypsin-like proteases of therapeutic relevance.

Keywords: anticoagulants; blood coagulation factors; protein engineering; thrombin; zymogens.

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Introduction

The application of therapeutic proteases to diseases of coagulation pathways is well documented [1]. In properly regulated cellular pathways, proteases are synthesized as inactive precursors, which are then activated in response to the metabolic state of the cell, and in response to extracellular events, such as vascular injury [2]. This biological strategy has been successfully applied for protease production in recombinant organisms by overexpression of proteases as inactive precursors, followed by an activation step. Among the first proteins manufactured by recombinant engineering were proteases of the coagulation cascade for hemophilia treatment [1]. Factor IX zymogen is overexpressed as a secretion construct in Chinese hamster ovary cells, where activation occurs by furin cleavage of the proenzyme [3]. Production of activated protein C was also accomplished by such a strategy, in which protein C zymogen is secreted into recombinant mammalian cell culture medium and subsequently activated by thrombin to the active protease [1].

The need for exogenous enzymes for the activation step in the production of therapeutic proteases presents challenges upon scale-up, including potential safety concerns associated with proteins isolated from tissues or blood products, maintenance of consistent quality and availability of the proteases, and increased cost of production. An alternative strategy for zymogen activation has emerged recently from the structural biology of thrombin precursors. Prethrombin-2 has Arg15 in the site of proteolytic activation by prothrombinase or ecarin, in electrostatic interaction with Glu14e, Asp14l and Glu18 [4]. Disruption of these interactions by mutagenesis produces derivatives that spontaneously convert to thrombin, without appreciable perturbation of the functional properties of the enzyme [5]. The reaction is started by the zymogen itself, and is abrogated by inactivation of the catalytic Ser195. This suggests a convenient strategy for the production of protein therapeutics with desired pharmacodynamic

properties that obviates the need for external enzymes and associated potential regulatory hurdles. Here, we describe a large-scale production strategy that exploits autoactivation for the thrombin mutant W215A/E217A (WE) [6], which is currently in preclinical development, owing to its compelling profile of efficacy and safety as an anticoagulant/antithrombotic and anti-inflammatory agent *in vivo*, as documented by several preclinical studies in rodent and non-human primate models [7–12]. The strategy offers a suitable alternative to existing protocols for the production of thrombin from activation of prethrombin-1 by prothrombinase [13,14] or prethrombin-2 by ecarin [15,16], thereby obviating the need for, costs of and possible contamination from external proteases.

Materials and methods

Purification of WEDGE was performed in a similar way to purification of WE expressed in *Escherichia coli* [4,16], with modifications for larger-scale production. Inclusion bodies were produced by the use of fed-batch fermentation, employing 2 × M9 yeast extract glucose medium and a glycerol plus yeast extract feed solution. Refolding was initiated by addition of reduced, denatured inclusion bodies to rapidly stirred refolding buffer. Concentration and diafiltration of refolding reactions prior to heparin–Sepharose chromatography were carried out with a hollow-fiber ultrafiltration cartridge. Autoactivation was allowed to proceed at room temperature and pH 8.0 after concentration of the heparin–Sepharose pool to 2–3 mg mL^{-1}. The progress of autoactivation was monitored by reversed-phase HPLC separation of thrombin A and B chains, and other intermediate forms, with a Vydac C4 2.1 × 50 mm column. After sample reduction in 3 M guanidine-HCl by 10 mM dithiothreitol, acidified samples were subjected to chromatography with gradient elution from 15% to 65% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.20 mL min^{-1} and UV detection at 214 nm. After autoactivation had reached completion, buffer exchange with a Sephadex G-25 column was followed by cation exchange chromatography on SP-Sepharose in MES buffer (pH 6.0) with elution of active WEDGE with a gradient of NaCl before storage of bulk WEDGE at − 20 °C. The overall yield from 14 g of inclusion bodies derived from 5 L of fermentation to final ~ 33 mg of bulk product was 1.2%, which is quite similar to the 1.1% yield of WE produced in *E. coli*. This yield is typical for refolding of proteins with multiple disulfide bonds. Samples for *in vivo* studies were treated with Detoxi-Gel (Thermo-Fisher, Waltham, MA, USA) before use to remove residual endotoxin. Studies with baboons were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Baboons were given a single intravenous bolus dose of WEDGE in 1 mL of saline. On day 1, baboon 1 received a single dose of 2.5 μg kg^{-1} WEDGE, and baboon 2 received a single dose of 1.0 μg kg^{-1} WEDGE. On day 2, baboon 2 received a single dose of 2.5 μg kg^{-1} WEDGE. The activated partial thromboplastin time (APTT) was measured after collection of blood samples into a 1 : 10 volume of 3.2% citrate buffer and then processed to platelet-poor plasma prior to treatment, and at 5, 15, 30, 60 and 120 min post-treatment. The APTT was read on a KC-1 with standard protocols. The effects of WEDGE were also assessed with the standard-template skin bleeding time test (Surgicutt; International Technidyne, Piscataway, NJ, USA) at 15, 30 and 60 min after the start of treatment.

Results and discussion

The production of several constructs of prethrombin-2 carrying the WE substitution with mutations in the activation domain revealed a construct carrying the additional three substitutions D14lA, G14mP and E18A (WEDGE) that was completely activated to thrombin in 10 h when used at a concentration of 3 mg mL^{-1}. This time frame is consistent with protocols for large-scale production in a biotechnological/pharmaceutical setting. WEDGE has functional activities towards physiologic

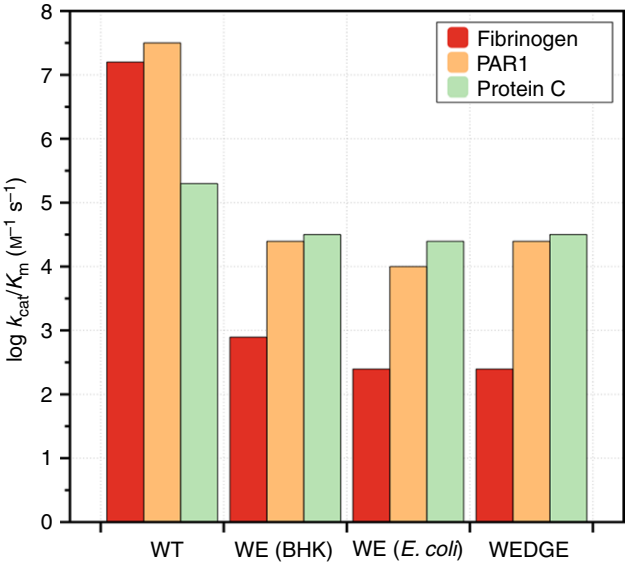

Fig. 1. Values of the log of the specificity constant k_{cat}/K_m (M^{-1} s^{-1}) for the hydrolysis of physiologic substrates (fibrinogen, protease-activated receptor 1 [PAR1] fragment ^{33}ATNATLDPRSFLLRNP NDKYEPFWEDEEKN^{62} and protein C in the presence of 10 nM thrombomodulin and 5 mM $CaCl_2$) by wild-type (WT) thrombin, W215A/E217A (WE) expressed in BHK cells as prethrombin-1 and activated with prothrombinase [6] or expressed in *Escherichia coli* as prethrombin-2 and activated with ecarin [16], and WEDGE produced by autoactivation. The WE mutation causes a drastic loss of activity towards fibrinogen and PAR1, but has only a modest effect on protein C activation in the presence of Ca^{2+} and thrombomodulin. The additional mutations introduced in WEDGE relative to WE to enable autoactivation are inconsequential with regard to the functional properties of the construct. Experimental conditions were as follows: 5 mM Tris, 0.1% PEG-8000 and 145 mM NaCl (pH 7.4) at 37 °C.

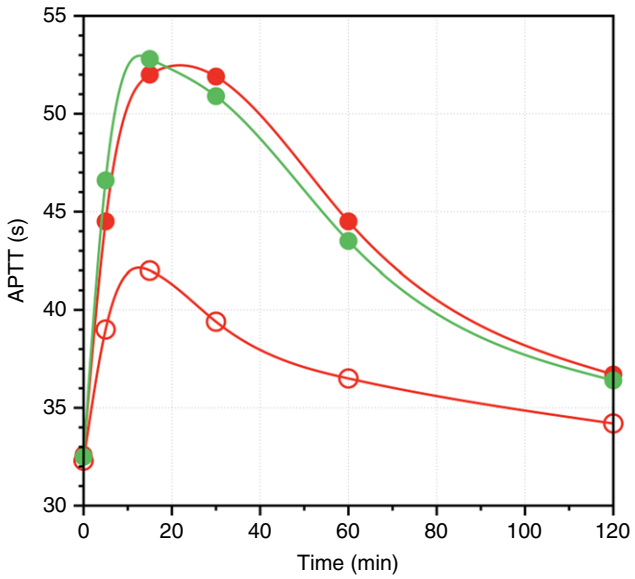

Fig. 2. Activated partial thromboplastin time (APTT) values as a function of time following a single bolus injection of WEDGE at 1.0 μg kg^{-1} (open red circles) and 2.5 μg kg^{-1} (closed red circles), or 2.5 μg kg^{-1} in a second baboon (closed green circles). WEDGE causes the APTT to increase significantly and transiently in a dose-dependent manner that reproduces the effect previously reported for W215A/E217A (WE) [9,10].

substrates *in vitro* (Fig. 1) that are practically identical to those reported for WE produced in mammalian BHK cells from prethrombin-1 and activated with prothrombinase [6], or produced in *E. coli* from prethrombin-2 and activated with ecarin [4,16]. Hence, differences in glycosylation and the introduction of three additional mutations in the activation domain of WEDGE relative to WE are inconsequential with regard to the kinetics of substrate hydrolysis.

The pharmacodynamic efficacy of WEDGE was tested in non-human primates by analysis of the APTT in plasma. Bolus administration of WEDGE caused prolongation of the APTT (Fig. 2), with the greatest effect occurring at 15 min post-injection, and with a return to baseline by 120 min. WEDGE prolonged the APTT approximately 1.6-fold at 2.5 μg kg^{-1} and 1.3-fold at 1.0 μg kg^{-1} after 15 min, consistent with the dose-dependent increase in the APTT reported previously for WE [10]. There were no WEDGE-related adverse events in the study subjects over the time scale of the experiments. Bleeding times measured at 15 min (3.3 min), 30 min (2.8 min) and 60 min (2.2 min) post-injection were not significantly elevated over baseline (2.5 min).

The development of an efficient strategy for the production of thrombin from its inactive precursors by autoactivation suggests other applications. The structurally related zymogen protein C has been extensively studied as an anticoagulant and anti-inflammatory agent [17–19]. The production of activated protein C requires thrombin, which must be added to the preparation and then eliminated to avoid potential thrombotic complications. The structural analogy of the activation domain of protein C with prethrombin-2 supports the viability of autoactivation for this natural anticoagulant factor [20] and its large-scale production devoid of external enzymes. In general, the activation sequence of a trypsin-like protease may be re-engineered to promote autoactivation for the production of mature proteases of clinical and biotechnological relevance.

Addendum

D. C. Wood, L. A. Pelc, N. Pozzi, A. Gruber, and E. Di Cera designed the research. D. C. Wood, A. Gruber, and E. Di Cera analyzed the data. D. C. Wood, L. A. Pelc, N. Pozzi, M. Wallisch, E. I. Tucker, and N. G. Verbout performed the research. D. C. Wood and E. Di Cera wrote the manuscript.

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Disclosure of Conflict of Interests

N. Pozzi and E. Di Cera have a patent pending on the autoactivation of trypsin-like proteases. The other authors state that they have no conflict of interest.

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