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Synthesis and characterization of novel fluorogenic substrates of coagulation factor XIII-A

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ABSTRACT

Further development of our recently published Glu(pNA)-containing peptides (Anal. Biochem. 428 (2012) 73–80) provided new fluorogenic substrates for the activated blood coagulation factor XIII. A first series was designed by incorporation of Glu(AMC) at the penultimate position from the N terminus. For the best derivative H-Tyr-Glu(AMC)-Val-Lys-Val-Ile-NH_2, a moderate k_{cat}/K_m value of 34 s^{-1} M^{-1} was determined, which is more than 100-fold reduced compared with the previously reported Glu(pNA) substrates. Furthermore, two fluorescence resonance energy transfer (FRET) substrates were prepared by incorporation of an *N*-methyl-anthraniloyl fluorophore and a 2,4-dinitrophenyl quencher. Both substrates were excellently cleaved by FXIII-A_2^*, which is generated from its zymogen by activation of thrombin in the presence of calcium ions. In the absence and presence of H-Gly-ethyl ester, k_{cat}/K_m values of 8010 and 8660 s^{-1} M^{-1}, respectively, were found for the conversion of H-Lys(*N*(Me)Abz)-Glu(NH-$(CH_2)_4$-NH-Dnp)-Val-Lys-Val-Ile-Gly-NH_2 (substrate **8**). These values are more than 200-fold improved compared with the Glu(AMC) substrates. Substrate **8** is suitable for the measurement of FXIII-A_2^* activities in plasma samples as well as for in vitro measurements. Furthermore, it was used for the determination of the inhibitory potency of a newly synthesized chloromethyl ketone derivative, Cbz-Phe-Glu(CMK)-Val-Lys-Val-Ile-Gly-NH_2, which was found to be a potent irreversible inhibitor of FXIII-A_2^*.

Activated human blood coagulation factor XIII [2] belongs to the family of transglutaminases and catalyzes an isopeptide bond formation between the side chain of a peptide-bound glutamine with a lysine side chain [1]. The main physiological role of activated FXIII is the stabilization of blood clots by crosslinking of fibrin and additional incorporation of the antifibrinolytic α_2-plasmin inhibitor (α_2-PI) into the fibrin polymer. Therefore, a pathological FXIII deficiency can cause severe bleeding complications [2]. Furthermore, additional diseases have been associated with altered FXIII levels [3], including cardiovascular [4,5] and gastrointestinal [6] disorders, as well as impaired wound healing [7]. Plasma FXIII (pFXIII) is a tetramer consisting of two potentially active A subunits and two carrier B subunits. In comparison with pFXIII, the cellular form (cFXIII) is a homodimer of two A subunits, which is present in the cytoplasm of platelets, megakaryocytes, monocytes, and monocyte-derived macrophages [8]. pFXIII is activated by thrombin, which cleaves the peptide bond between Arg37 and Gly38, releasing the 4-kDa activation peptide AP-FXIII from the N-terminus of the A subunit. Afterward, the B subunits dissociate in the presence of Ca^{2+} ions, providing FXIII-A_2^* in its active configuration (the abbreviations used for the different forms of FXIII follow the recommended literature [9]). The Ca^{2+}-induced structural change unmasks the active site cysteine, which is not accessible in the inactive form [10].

Various methods have been developed for the determination of FXIII-A_2^* activity [11]. Most of them use the endogenous transglutaminase (TGase) activity under release of ammonia from natural glutamine residues or its isopeptidase activity, resulting in the liberation of primary amines from the side chain of synthetic alkylated glutamine derivatives. In 1991, Fickenscher and coworkers introduced a coupled photometric assay based on the detection of ammonia, which is released from a synthetic decapeptide derived from β-casein [12]. Based on the isopeptidase activity of FXIII-A_2^*, Lorand and coworkers developed a fluorescence

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[2] *Abbreviations used:* FXIII, factor XIII; α_2-PI, α_2-plasmin inhibitor; pFXIII, plasma FXIII; cFXIII, cellular FXIII; FXIII-A_2^*, active form of factor XIII generated by thrombin in presence of Ca^{2+} ions; TGase, transglutaminase; FRET, fluorescence resonance energy transfer; Cad, cadaverine; pNA, *p*-nitroanilide; AMC, 7-amino-4-methylcoumarin; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; Boc, *tert*-butyloxycarbonyl; Abz, 2-aminobenzoyl; DIPEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; OSu, succinimidooxy; MS, mass spectrometry; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; Put-Dnp, *N*-(2,4-dinitrophenyl)putrescine; Cbz, benzyloxycarbonyl; CMK, chloromethyl ketone; THF, tetrahydrofuran; BOP, benzotriazolyl-1-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HBTU, *O*-(benzotriazol-1-yl)-tetramethyluronium hexafluorophosphate; PyBroP, bromotripyrrolidinophosphonium hexafluorophosphate; RFU, relative fluorescence units; *N*(Me)Abz, *N*-methyl-anthraniloyl; Put, putrescine; Dnp, 2,4-dinitrophenyl; Cad-Dnp, *N*-(2,4-dinitrophenyl)cadaverine.

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resonance energy transfer (FRET) assay using a synthetic hexapeptide substrate containing a dansyl or anthraniloyl fluorophore at the N-terminus and a 2,4-dinitrophenyl quenching group linked via a cadaverine (Cad) spacer to the side chain of glutamate [13]. However, relatively high enzyme concentrations above 0.5 μM needed to be used for measurements with this substrate. The sensitivity of this test system was improved by using a more suitable analogue derived from the N-terminal sequence of α_2-PI. This substrate possesses a K_m value of 19.8 μM and enables measurements over a broad FXIII-A_2* concentration range between 0.05 and 8.8 IU/ml [14]. However, a relatively large 12-mer peptide sequence was required to achieve sufficient conversion rates.

Therefore, we tried to develop shorter fluorogenic substrates with sufficient sensitivity suitable for the determination of the FXIII-A_2* activity in plasma that were derived from our recently published Glu(pNA)-containing heptapeptides [15]. In the first series, the pNA (*p*-nitroanilide) chromophore was replaced by an amidomethylcoumarin (AMC), known as a suitable fluorophore in substrates of the related tissue TGase 2 [16] and γ-glutamyltranspeptidase [17,18]. In our previous study, we found two factors that have a strong impact on the substrate properties for FXIII-A_2*. The Glu(pNA) residue should be incorporated at the second position of the sequence, and the N-terminus of the peptide should be unprotected. Therefore, in contrast to the previously developed FRET substrates [13,14], we designed new analogues where the fluorophore was moved to a suitable side chain of the peptide maintaining a free N-terminus. One of the new FRET substrates enabled convenient FXIII-A_2* activity measurements in plasma and was used for the determination of the inhibitory potency of a newly synthesized chloromethyl ketone inhibitor. The results of these experiments are summarized in this publication.

Materials and methods

Synthesis

Analytical high-performance liquid chromatography (HPLC) experiments were performed on a Shimadzu LC-10A system (column: Nucleodur C_{18}, 5 μm, 100 Å, 4.6 × 250 mm, Macherey–Nagel, Düren, Germany) with a linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) (1–90% in 89 min and detection at 220 nm) at a flow rate of 1 ml/min. The final substrates were purified by preparative HPLC (pumps: Varian PrepStar model 218 gradient system; detector: ProStar model 320; fraction collector: Varian model 701) using a C_8 column (Nucleodur, 5 μm, 100 Å, 32 × 250 mm, Macherey–Nagel) and a linear gradient of acetonitrile (50% increase of the acetonitrile concentration within 100 min, with the used starting concentration of acetonitrile depending on the hydrophobicity of the individual compounds) containing 0.1% TFA at a flow rate of 20 ml/min. All peptides were finally obtained as TFA salts after lyophilization. The molecular mass of the synthesized compounds was determined using a QTrap 2000 electrospray ionization (ESI) spectrometer (Applied Biosystems).

All reagents for synthesis, including standard 9-fluorenylmethoxycarbonyl (Fmoc) amino acids, coupling reagents, resins, solvents, and all other reagents, were obtained from Orpegen, Bachem, Iris, Fluka, Acros, or Aldrich. Fmoc-Lys(*N*(Me), *N*(Boc)Abz)-OH was purchased from Bachem.

Solution synthesis of starting materials

Fmoc-Glu(AMC)-OH

First, 1.627 g (4.468 mmol) of H-Glu(AMC)-OH [18] and 0.783 ml (4.5 mmol) of *N,N*-diisopropylethylamine (DIPEA) were suspended in 100 ml of dimethylformamide (DMF) and 50 ml of water at 0 °C. Next, the mixture was treated with 1.507 g (4.468 mmol) of Fmoc-OSu dissolved in 20 ml of acetonitrile and was stirred for 30 min under ice cooling, followed by additional treatment with 0.35 ml (2 mmol) of DIPEA. The mixture was stirred at room temperature overnight. The solvent was removed in vacuo, and the remaining residue was dissolved in ethyl acetate and washed three times with 5% $KHSO_4$ solution and three times with brine. The organic phase was dried over Na_2SO_4, the solvent was removed in vacuo, and the remaining solid was recrystallized from ethyl acetate (slightly yellow solid; yield: 2.2 g, 4.17 mmol; HPLC: 56.3 min; MS: calc. 526.17, found 527.11 $(M+H)^+$ and 525.21 $(M-H)^-$; NMR: ^{13}C NMR (DMSO-d_6, 100 Mhz): δ [ppm] = 173.55, 171.14, 160.01, 156.15, 153.66, 153.04, 143.80, 143.76, 142.50, 140.67, 127.60, 127.04, 125.80, 125.22, 120.05, 115.03, 114.79, 112.09, 105.44, 65.67, 53.33, 46.63, 32.93, 26.20, 17.92).

Fmoc-Glu(Put-Dnp)-OH

After 0.500 g (1.175 mmol) of Fmoc-Glu-OtBu was dissolved in 10 ml of DMF at −20 °C, the mixture was treated with 0.129 ml (1.175 mmol) of *N*-methylmorpholine and 0.153 ml (1.175 mmol) of isobutyl chloroformate. After 10 min, 0.376 g (1.293 mmol) of *N*-(2,4-dinitrophenyl)butane-1,4-diamine was added and the mixture was stirred for 60 min at −20 °C in the dark and at room temperature overnight. The solvent was removed in vacuo (yellow oil; HPLC: 82.7 min; MS: calc. 661.27, found 662.33 $(M+H)^+$). The remaining oil was treated with 10 ml of TFA and stirred for 2 h. The remaining residue was solved in ethyl acetate and washed three times with brine. The organic phase was dried over Na_2SO_4, and the solvent was removed in vacuo (yellow oil; HPLC: 72.7 min; MS: calc. 605.21, found 606.30 $(M+H)^+$; NMR: ^{13}C NMR (DMSO-d_6, 126 MHz): δ [ppm] = 173.7, 171.1, 156.1, 148.1, 143.8, 140.6, 134.6, 129.9, 129.6, 127.6, 127.0, 125.2, 123.6, 120.0, 115.2, 65.6, 53.4, 46.6, 42.5, 37.9, 31.7, 26.8, 26.4, 25.5).

Cbz-Phe-Glu-OtBu

First, 0.850 g (4.18 mmol) of H-Glu-OtBu was suspended in 60 ml of dry dichloromethane and treated with 1.16 ml of trimethylsilyl chloride (9.2 mmol) and 1.6 ml (9.2 mmol) of DIPEA. Then, the mixture was refluxed for 30 min. At 0 °C, 1.65 g (4.18 mmol) of Cbz-Phe-OSu was added to the clear solution over a period of 10 min, followed by additional treatment with 0.72 ml (4.18 mmol) of DIPEA. The mixture was stirred for 30 min under ice cooling and at room temperature overnight. The solvent was removed in vacuo, and the remaining residue was dissolved in ethyl acetate, washed three times with 5% $KHSO_4$ and three times with brine, dried over Na_2SO_4, and filtrated, and the solvent was evaporated (white solid; yield: 1.84 g, 3.81 mmol; HPLC: 57.3 min; MS: calc. 484.2, found 485.3 $(M+H)^+$; NMR: ^{13}C NMR (DMSO-d_6, 126 MHz): δ [ppm] = 173.6, 171.7, 170.7, 155.8, 138.0, 136.9, 129.1, 128.2, 128.0, 127.6, 127.4, 126.2, 80.6, 65.1, 55.8, 52.0, 37.4, 29.9, 27.5, 26.2).

Cbz-Phe-Glu(CMK)-OH

After 1.840 g (3.81 mmol) of Cbz-Phe-Glu-OtBu was dissolved in dry tetrahydrofuran (THF), the solution was cooled to −20 °C and 0.52 ml (4.00 mmol) of isobutyl chloroformate was added, followed by additional treatment with 0.44 ml (4.00 mmol) of *N*-methylmorpholine. The mixture was stirred for 10 min, and afterward it was filtered into a Schlenk flask and cooled to −70 °C. The diazomethane was generated by adding 2 ml of 70% KOH solution at 0 °C to a stirred suspension of 1.1 g (5.13 mmol) of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (diazald) in a mixture of 10 ml of ether, 0.5 ml of water, 1.5 ml of ethanol, and 2.5 ml of 2-methoxyethanol [19]. The diazomethane was distilled at 35 to 40 °C into the cooled (−70 °C) mixed anhydride solution,