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Oxidation-induced modifications of the catalytic subunits of plasma fibrin-stabilizing factor at the different stages of its activation identified by mass spectrometry

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ABSTRACT

Plasma fibrin-stabilizing factor (pFXIII) is a heterotetrameric proenzyme composed of two catalytic A subunits (FXIII-A_2) and two inhibitory/carrier B subunits (FXIII-B_2). The main function of the protein is the formation of cross-links between the polypeptide chains of the fibrin clot. The conversion of pFXIII into the enzymatic form FXIII-A_2* is a multistage process. Like many other blood plasma proteins, pFXIII is an oxidant-susceptible target. The influence of distinct sites susceptible to oxidation-mediated modifications on the changes in the structural-functional characteristics of the protein remains fully unexplored. For the first time, a set of the oxidation sites within FXIII-A_2 under ozone-induced oxidation of pFXIII at different stages of its activation have been identified by mass spectrometry, and the extent as well as the chemical nature of these modifications have been explored. It was shown that the set of amino acid residues susceptible to oxidative attack and the degree of oxidation of these residues in FXIII-A_2 of non-activated pFXIII, pFXIII activated by Ca^{2+} and fully activated pFXIII treated with thrombin and Ca^{2+} significantly differ. The obtained data enable one to postulate that in the process of the proenzyme conversion into FXIII-A_2*, new earlier-unexposed amino acid residues become available for the oxidizer while some of the initially surface-exhibited residues are buried within the protein globule.

1. Introduction

Plasma fibrin-stabilizing factor (pFXIII) is a member of the transglutaminase family (*endo*-γ-glutamine: ε-lysine transferase) that is capable of producing covalent bonds between protein chains. The role of pFXIII in hemostasis is well known to lie in the stabilization of the fibrin clot and protection of it from fibrinolytic degradation [1,2]. Besides this, pFXIII has many more potentially important functions beyond coagulation [3,4].

pFXIII exists in the circulation system as a heterotetrameric proenzyme FXIII-A_2B_2, which consists of two single-stranded catalytic A subunits (FXIII-A_2) each having the molecular weight of ~83 kDa, and two identical single-stranded inhibitory/carrier B subunits (FXIII-B_2) with the molecular weight of ~80 kDa each. The subunits are held together by weak non-covalent bonds [1,5].

The catalytic FXIII-A subunit is a single polypeptide chain of 731 residues including nine cysteine residues, none of which forms disulfide bonds. The polypeptide chain is folded into five distinct structural domains: the 37 amino acid N-terminal activation peptide (FXIII-AP), β-sandwich (Gly38-184Phe), the catalytic core domain (Asn185-515Arg) divided into NH_2- (189–332) and COOH-terminal (333–515) subdomains, β-barrel 1 (Ser516-628Thr), and β-barrel 2 (Ile629-731Met) [1,5–7]. The active site is comprised of residues Cys314, His373, and Asp396 inherent to the catalytic triad of other cysteine proteases.

The non-catalytic FXIII-B subunit consisting of 641 amino acids is a mosaic protein composed of ten short tandem structures, designated as Sushi domains, each of which is formed by approximately 60 amino acid residues with no free cysteine groups. The domains are linked together by a pair of internal disulfide bonds [8].

The conversion of FXIII-A_2B_2 into the active form of the enzyme (EC 2.3.2.13) is a multistage process that is physiologically induced in the final phase of the clotting cascade by the joint action of thrombin and Ca^{2+}. The first stage is the thrombin-catalyzed proteolytic cleavage of the Arg37-Gly38 bond at the amino-terminus of the FXIII-A subunit,

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leading to the release of the activation peptide, FXIII-AP, and conversion of the FXIII-A_2B_2 heterotetramer into the FXIII-$A_2'B_2$ heterotetramer, which also does not have enzymatic activity. The second stage of activation requires calcium ions in order to dissociate the FXIII-$A_2'B_2$ heterotetramer with the formation of FXIII-A_2' and FXIII-B_2 subunits. At the last stage, FXIII-A_2', in the presence of Ca^{2+}, undergoes large-scale conformational changes resulting in the exposition of residues Cys314, His373, and Asp396 leading to the formation of the enzyme FXIII-A_2* [1,9].

Like many other blood plasma proteins, the plasma fibrin-stabilizing factor is an oxidant-susceptible target. Currently, there is a limited number of studies on oxidative modifications of this protein [10–13]. Ozone-induced oxidation of pFXIII was found to affect the enzymatic activity of FXIIIa which is greatly dependent on the stage of the zymogen transformation at which oxidation was carried out. Chemical transformation of aromatic, NH, SH and S—S groups evidenced by FTIR- and Raman spectroscopy demonstrates the oxidation of amino acid residues in the FXIII polypeptide chains. High resolution mass spectrometry approach is promising for characterization of oxidative protein modifications by reactive oxygen species (ROS) [14,15]. Conversion of pFXIII to the enzymatic form proved to increase the protein vulnerability to oxidation in the order: FXIII-A_2B_2 < FXIII-$A_2'B_2$ < FXIII-A_2B_2 + Ca^{2+} < FXIII-A_2* + FXIII-B_2 [10,11]. Recently, by applying mass-spectrometric methods it was shown that ozone-mediated modification of pFXIII the oxidation sites are scattered along all of the structural elements (SE's) of the catalytic FXIII-A subunit over all of its structural elements [13]. The greatest number of oxidative alterations involves the most readily oxidizable sulfur-containing amino acid residues of Cys and Met as well as aromatic amino acid residues of Tyr and Trp. Nevertheless, pFXIII still remains an extremely poorly explored protein in the aspect of determining the amino acid residues that undergo oxidative modifications during enzymatic activation. Therefore, the purpose of this study is to identify amino acid residues undergoing oxidation in the FXIII-A subunits of pFXIII under induced oxidative conditions at different stages of proenzyme activation as well as the extent and the nature of these chemical modifications. Identification of oxidized amino acid residues located in the catalytic subunit may provide much-needed information of the mechanism of pFXIII damage and the decrease in enzymatic activity due to oxidation. In addition, it allows to make certain conclusions both about spatial accessibility of amino acid residues in FXIII-A for ROS and structural rearrangements within the oxidized protein.

2. Materials and methods

Human blood plasma was obtained from the Moscow Central Station for Blood Transfusion. DEAE-ToyoPearl M650 was purchased from Tosoh (Japan). Trypsin Gold (mass spectrometry grade, V5280) and Glu-C (sequencing grade, V1651) were obtained from Promega (USA). All other chemicals were of analytical-grade or higher. All solutions were prepared with Milli-Q water.

2.1. Isolation of pFXIII

pFXIII was isolated from human blood plasma by a fractional precipitation procedure with ammonium sulphate and subsequent ion exchange chromatography on DEAE-ToyoPearl M650 [16]. The transformation of pFXIII into FXIIIa in the presence of thrombin and calcium ions was accomplished as reported before [17].

A series of pFXIII samples with a different prehistory has been studied. Their codes are: pristine proenzyme pFXIII (FXIII-A_2B_2); oxidized proenzyme pFXIII (oxFXIII-A_2B_2); pFXIII treated with a 5 mMCa^{2+} solution (FXIII-A_2B_2 + Ca^{2+}); oxidized pFXIII treated with a 5 mMCa^{2+} solution (oxFXIII-A_2B_2 + Ca^{2+}); FXIIIa prepared by activation of pFXIII with thrombin in the presence of 5 mM Ca^{2+} (FXIII-A_2*) and oxidized FXIIIa (oxFXIII-A_2*).

2.2. Oxidation of fibrin-stabilizing factor

The solution of fibrin-stabilizing factor (1.0 mg/ml) in Tris-HCl (150 mM) buffer with 0.15 M NaCl (pH 7.4) was ozonized in a quartz reactor (5.5 ml) by blowing the ozone–oxygen mixture through the reactor [18]. The full exhaustion of ozone in each experiment was confirmed by spectrophotometric measurements at 254 nm. The amount of oxidation agent was equal to 1×10^{-6} mol per 1 mg of protein.

2.3. Enzymatic digests

The samples were digested with Glu-C and trypsin. The protein was diluted in 50 mM Tris-HCl buffer with 0.15 M NaCl (pH 8.0) and hydrolyzed with Glu-C and trypsin consistently at an enzyme:protein ratio of 1:50 mixture (w/w) overnight and for 16 h respectively. The reaction was stopped by adding formic acid to a final concentration of 0.1%.

2.4. HPLC-MS/MS analysis

HPLC-MS/MS experiments were performed on an Agilent 1100 nanoLC (Agilent Technologies Inc., Santa Clara, USA) coupled to 7T LTQ-FT Ultra (Thermo, Bremen, Germany) high resolution mass spectrometer.

For chromatographic separation 1 μl of each sample was injected to homemade C18 column (75 μm × 12 cm, Reprosil-Pur Basic C18, 3 μm; Dr. Maisch HPLC GmbH, ammerbuch-Entringen, Germany) made by the method described by Mann et al. [19]. The mobile phase used: solvent A: 0.1% formic acid in H_2O; solvent B: acetonitrile. Chromatography was performed with a linear gradient increasing the relative content of solvent B from 3% to 50% in 45 min.

The ion spray voltage was set to 2.8 kV. Mass spectrometric analysis of the peptide fractions was performed using the Xcalibur software (Thermo Electron, Bremen, Germany) with automatic spectra measurement in a 2-stage mode. At the first stage the accurate masses of peptides were measured in the ICR cell in the range m/z = 300–1600 with resolution R = 50,000 at m/z = 400 (the number of ions in the ICR cell was set to 5×10^6). At the second step five most intensive peaks from the first stage were subjected to collision-induced dissociation (CID) and fragment spectra were registered in the linear ion trap (number of ions in the LTQ was set to 3×10^4). After fragmentation the corresponding parent masses were dynamically excluded from consideration for the next 30 s.

For each of the samples three technical replicates of mass spectrometry measurements were performed.

2.5. Data processing

Peptides of the catalytic subunit FXIII obtained as the result of Glu-C and tryptic cleavage were identified by searching the against sequence of F13A_HUMAN (P00488) from UniProtKB database using PEAKS Studio software (v. 8.0, Bioinformatics Solutions Inc., Waterloo, ON, Canada). Non-specific cleavage was allowed at one terminus; mass accuracy for the precursor ion was set to 15 ppm; mass accuracy for MS/MS fragments to 0.5 Da. The cut-off false discovery rate (FDR) for peptides was set to 0.1%. Peptides with minimum length of 5 amino acids were considered for identification with maximum allowed of 3 variable PTMs per peptide according to the recommendations given in [20,21]. The list of modifications specified in the calculation is given in Table 1.

The relative content of oxidized AARs was calculated by dividing the number of modified residues by the whole number of residues in the corresponding sequence, and converted to percentage.

The oxidation degree (OD) of all modification types for each amino acid residue (AAR) was calculated as the sum of the peptide areas for the determined oxidative modification of this AAR, normalized by the